UNIVERSITY OF ALBERTA

AVIAN ISOANTIGENS

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by

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The undersigned certify that they have read and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Avian Isoantigens", submitted by Lyle A. David, B.Sc., in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

The B isoantigens of chickens are present in the external surface and within the cytoplasm of cells of the hemopoietic system. The distribution of the surface antigen is very similar to the distribution of the H-2 isoantigens of mice. Some of the B isoantigen within the cytoplasm is concentrated in droplets which resemble secretory droplets. This distribution may be more closely related to the distribution of A, B, and H isoantigens in older human embryos. The conditions under which the B isoantigens of chickens are visualized by immunofluorescence suggest that the specific, determinative structures of the transplantation isoantigens are not amino acids, peptides, or proteins.

This report embodies five distinct contributions; (1) the extension of immunofluorescent studies of transplantation isoantigens to a species other than the mouse, (2) the confirmation with fluorescent isoantibodies of interpretations based on fluorescent anti-globulin antibodies, (3) the demonstration of intracellular droplets of transplantation isoantigen, (4) the demonstration that ethanol fixation interferes with the immunofluorescent visualization of transplantation isoantigens, and (5) the demonstration that formalin fixation enhances the immunofluorescent visualization of transplantation isoantigens.

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INTRODUCTION

Before the turn of the century, antigens were considered to be foreign substances, such as bacteria, which elicited, and reacted with, specific antibodies when these antigens penetrated the physiological defences of an animal. Such antigen-antibody reactions were recognized as a fundamental aspect of immunity to disease.

In 1900 Karl Landsteiner discovered that human red blood cells could be divided into four groups on the basis of agglutination tests. Two substances called A and B were found to be distributed on red cells so that an individual might possess A, B, AB or neither (0). When either of the substances was lacking in an individual the corresponding agglutinin was present in his serum. The presence of the A and B substances was dependent on the inheritance of two dominant genes at a single locus. Although the agglutinins were present in sera without prior stimulation, it was recognized that the A and B substances were antigens and the agglutination of A and B cells was a specific antigen-antibody reaction.

This discovery made it possible to demonstrate inherited difference among individuals of the same species by immunological tests. The concept of immunity could be enlarged to include isoantigens: structural components or products of cells, which are immunologically foreign to other members of the same species, and the inheritance of which is controlled by two or more alleles. The corresponding isoantibodies may be present as natural antibodies in the serum of individuals not possessing the isoantigen, as in the ABO system, or they may appear only after immunization, as in the classical immune response.

By 1913 there was a general awareness that the rejection of tumors and normal tissue transplanted to other animals was due to an immune response of the host to the foreign tissue. It was recognized that the barriers which prevented

acceptance were greater between species than within. Varieties within a species could also be segregated on the basis of acceptance or rejection of transplanted tumors. White mice would accept tumors from some other white mice but the tumors would die if transplanted to black or brown mice (Murphy, 1913). In 1927 Loeb and Wright established that tissue transplant rejection had a genetic basis and was due to inherited units of "individuality differential" present in the donor and absent in the host. They achieved this by demonstrating that closely related members of an inbred line of guinea pigs would accept skin grafts from each other. The more distant the relationship, as between inbred lines, the more severe was the rejection reaction. The F_1 hybrid resulting from crossing two inbred lines reacted weakly or not at all to skin from either parent but the parents reacted strongly to skin grafts from their F_1 offspring. Loeb and Wright did not, however, identify any of the presumed isoantigens or show the manner of their inheritance.

Such an identification was achieved by Peter Gorer in 1942 when he discovered a set of isoantigens controlled by the H-2 locus in the mouse. These isoantigens, under control of a series of codominant alleles, are present in many tissues and on the surface of erythrocytes. They were first detected by agglutination of erythrocytes using isoantibodies produced by immunization within the species, natural isoantibodies not being present. When a tumor was transplanted from a mouse possessing an H-2 isoantigen to a host which did not possess the same isoantigen, the tumor was destroyed and the animal survived. Subsequently, it was demonstrated that the H-2 locus exerts the same control over the rejection of skin grafts that it exercises in restraining the growth of tumors (Counce, et al. 1956). There is, however, a difference in the rejection of a skin graft and the suppression of a tumor. Immunity to some tumors seems to be related to the presence of circulating isoantibodies. The rejection of a skin graft is less dependent upon circulating isoantibodies.

Although skin graft rejection is a different expression of the immune response, there is no doubt that graft rejection is also due to the presence of isoantigens under genetic control. Not only the H-2 locus, but also the B locus of the chicken, control codominant isoantigenic systems demonstrable by agglutination and skin graft tests.

These, however, are exceptions. Most isoantigenic systems control transplantation isoantigens or erythrocyte isoantigens but not both. There is mounting evidence that some transplantation isoantigens are present not only in skin but in a variety of tissues and cells, especially leukocytes. A clear-cut demonstration of the distribution of transplantation isoantigens depends on new techniques being used. The technique we chose is the immunofluorescent technique of Albert Coons (1951).

Immunofluorescence has not been widely applied to the demonstration of isoantigens, due to technical difficulties. However, human blood group substances of the ABO and Rh systems have been demonstrated on agglutinated red cells (Jankovic, 1959) and on the surfaces of minor cell populations of fetal variant cells in the circulation of pregnant mothers (Cohen et al. 1960). The A, B and H substances have been charted in the tissues of human fetuses from three months of age by Szulman (1964) using fluorescent antisera. Jankovic and Lincoln (1959) demonstrated Rh (D) substance on leukocytes. This technique has also been applied to the H-2 system of the mouse (Möller, 1961). He reports specific attachment of H-2 isoantibody to erythrocytes, lymph node cells, bone marrow cells, epithelial cells and tumor cells.

The chicken, which has a well defined isoantigenic system, comparable to the H-2 locus, is the species we have selected. Codominant alleles at the B locus of chickens control isoantigens which provoke the formation of red cell agglutinating isoantibodies (Briles et al. 1950) and act as potent transplantation

isoantigens (Schierman and Nordskog, 1961). There are four such alleles present in the flock used in this study, each detectable by an agglutination reaction.

We chose the chicken because, among other reasons, it has several practical advantages. The genetics of the chicken has received much attention in recent years and large stocks of inbred and crossbred families are obtainable. Agglutination tests provide a simple method of distinguishing B and other genotypes. This makes it possible to study an isoantigenic system in heterozygous hybrid chickens, which are more viable than inbred chickens. Also, chickens are relatively easy to immunize and large amounts of antisera can be obtained.

Despite their importance, very little is known about the transplantation isoantigens. The present study is a step towards a better understanding of the embryonic origin and the intracellular distribution of the transplantation isoantigens. The species and technique are particularly well suited to this purpose.

MATERIALS AND METHODS

I. General Description of Chickens

All chickens used in this experiment were derived from a cross of two highly inbred lines maintained by the Hy-Line Poultry Farms, Johnston, Iowa. The hybrid eggs were hatched in a Jamesway Model 252 incubator at a constant temperature of 100 $\pm$ 0.2°F and 80% relative humidity reading. The eggs were turned automatically every six hours during the incubation period. On the twentieth day of incubation the eggs were segregated according to genotype and transferred to hatching trays. On the twenty-second day the newly hatched chicks were removed from the incubator and tagged on each wing with numbered wing tags as a permanent means of identification. The chicks were then placed in a Petersime model 25D. electrically heated brooder for a period of approximately six weeks. During this period they had free access to water and chick starter. The chickens were then moved to developer cages (model D825 Harford Eng. Co., Aberdeen, Md.) and fed laying ration. At approximately two months of age each bird was fitted with a 2-inch square plastic wing badge bearing up to three numbers which remained upright and permitted quick identification without unnecessary handling. The chickens were then transferred to laying cages (model 28, Harford Eng. Co., Aberdeen, Md.) or to open breeding pens.

Each chicken is identified by three sets of numbers: the first two sets, separated by an oblique, represent the metal wing tags; the last set, separated by a dash represents the plastic wing badge, i.e. 1234/1235-100.

II. Terminology

The terminology used to describe histocompatibility genes, isoantigens and isoantibodies is a modification of that of Briles, Allen and Millen (1957). Capital letters are used as symbols for genetic loci. Superscript numbers represent alleles and subscript numbers represent isoantigens controlled by the corresponding alleles. Isoantisera

are designated by the prefix "anti" plus the antigen which elicited the response. For example, the designation B^1B^2 would represent a chicken which carries alleles 1 and 2 at the B locus. The isoantigens involved would be written B_1B_2 and the homologous isoantiserum, anti B_1B_2. This differs from the terminology of Briles, Allen and Millen in that they would refer to the isoantiserum as B1B2.

III. Genotypic Description of Chickens

There are four alleles which may be found at the B isoantigenic locus in our stock: B^1, B^2, B^{13} and B^{14}. The basic bird is a B^2B^{14}, F_1 hybrid, obtained by crossing two inbred lines. The skin grafts to B^2B^{14} chicks were from B^1B^{14}, B^1B^{13}, B^2B^{13} and B^2B^{14} F_1 hybrid donors. In these birds there are six other isoantigenic loci which can be recognized by red cell agglutination. All skin graft donors were identical heterozygotes at these six other loci.

For the preparation of B isoantisera, B^2B^{14} F_1 hybrids were crossed to produce B^2B^2, B^2B^{14} and $B^{14}B^{14}$ F_2 hybrids with no more than two alleles segregating at each of the other six isoantigenic loci. In order to afford a constant supply of B^2B^2 and $B^{14}B^{14}$ chickens, two breeding groups were maintained. These consisted of six B^2B^2 males with twenty B^2B^2 females and four $B^{14}B^{14}$ males with twelve $B^{14}B^{14}$ females. Matings within the groups were at random. B^2B^{14} stocks were maintained by hatching eggs derived from crossing the original inbred lines.

IV. Skin Grafting

Mature skin was grafted to young chicks in the following manner: approximately twenty-four two day old chicks were anaesthetized by an intra-peritoneal (I.P.) injection of 0.10 mls. of Combuthal (30 mgm. of 1 part nembutal to 3 parts sodium pentothal per ml. of distilled H_2O). The chicks were then placed on a warm heating pad until unconscious. The down was plucked from their backs and the area swabbed with collodion. A 2 inch

square of gauze, wet with more collodion, was placed on this area and allowed to dry. A 1 cm. square of stiffened skin and gauze was cut from each side of the back using scissors and forceps. The right-hand graft was turned 180° and placed on the left as a control autograft. A full thickness homograft of mature skin was placed on the right side with its orientation also reversed so that feather growth would be forward.

The mature birds providing homografts were anaesthetized by I.P. injection of Nembutal (60 mgm. pentabarbitone sodium per ml. of distilled H_2O. An injection of 0.10 ml. per 100 gm. of body weight was usually adequate. The area of skin overlying the side of the rib cage was covered with gauze and collodion and allowed to dry. Grafts were removed one at a time and placed directly on the recipient chicks. The wound was closed with surgical clips and the area bandaged. An I.P. injection of 150,000 international units of penicillin G procaine in aqueous suspension (0.5 ml.) was given to protect against infection.

V. Production of Specific Isoantisera

Isoimmunization

In order to obtain cells for isoimmunization, up to 9 ml. of peripheral whole blood was drawn from the exposed cubital vein which lies on the underside of the wing of the chicken. A 10 ml. glass syringe, fitted with a #20 needle and containing 1 ml. of 10% sodium citrate was inserted into the vein at or near the base of the wing while the chicken was held on its side by another operator. By applying constant negative pressure to the syringe, 9 ml. of blood could be obtained in approximately 90 seconds, depending on the size of the vein. On removal of the needle, blood flow ceased after about a minute if the vein was squeezed off. Birds could be bled in this fashion, on alternate wings, every three days if necessary. Sterile procedures were not employed. All blood samples were refrigerated at 4°C until use.

The citrated whole blood was centrifuged at 2000 r.p.m. in a Servall RC2

refrigerated centrifuge for 20 minutes, in 50 ml plastic tubes, at a temperature of 4°C, to yield approximately 4 ml. of packed cells per 9 ml. of whole blood. The supernatant plasma was removed by vacuum suction into a trap flask and discarded. The packed cells were then washed free of protein by adding Alsever's (modified) solution in sufficient quantity to fill the tubes, followed by agitation on a Vortex mixer until all cells were in suspension. The cells were recentrifuged and rewashed twice. After the final centrifugation a 50% suspension of washed cells in Alsever's solution was prepared and used for intravenous injection. Alsever's solution was used for washing as clotting and hemolysis of cells sometimes occurred if isotonic saline solution alone was employed. This did not occur with Alsever's solution. Sodium azide was substituted for merthiolate in the preparation of Alsever's solution as it proved to be a more suitable preservative for serological material (Lachmann, 1965).

Highest titres of isoagglutinins were obtained by selecting birds which showed a good initial response to isoimmunization. Repeated intravenous injections of 1 ml. of a 50% suspension of cells in Alsever's solution were given approximately every seven days for two to three months.

Serum was obtained by bleeding with a dry syringe, without anticoagulant, after a 24 hour starvation period to reduce serum lipid content. After standing for 24 hours the clotted cells were centrifuged out at 5000 r.p.m. for twenty minutes and the serum decanted into rubber stoppered serum bottles for storage.

Three methods were evaluated in attempting to produce a specific isoantiserum, that is, a reagent that would react with a single B antigen and show no cross reactivity.

1. Adsorption

The first method was adsorption of isoantiserum with blood cells carrying the isoantigen(s) with which the isoantiserum cross-reacted (Briles, McGibbon and Irwin, 1950).

An isoantiserum produced by chickens immunized against B_2 or B_{14} cells was

tested by agglutination using B_1, B_2, B_{13} and B_{14} washed cells. If it agglutinated any type of cells, other than the type used for immunization, the isoantiserum was adsorbed with cells of the genotype it cross reacted with. A pool of washed cells was prepared by mixing cells from two to four birds. After the final centrifugation all supernatant was removed to avoid dilution of the isoantiserum. The isoantiserum was added to the packed cells and allowed to agglutinate for 2 hours under constant agitation at room temperature. Complete exhaustion of cross reactivity could be obtained without significant loss of strength of the isoantiserum, if large enough numbers of cells were used. As much as 20 ml. of packed cells per ml. of serum were sometimes necessary for complete adsorption. Usually, however, 2 ml. of cells were sufficient to adsorb the cross reactivity from 1 ml. of serum.

2. Preadsorption

The second method employed was called preadsorption. This method consisted of selecting donor - recipient pairs which provided a situation where B cross reactivity could not occur or was limited. For example, if a B^1B^{14} chicken is immunized with B_2B_2 cells it should produce anti-B_2 serum which could not react with B_1 or B_{14} antigens, as they are components of the immunized chicken's own antigenic system.

Chickens were selected for isoimmunization on the basis of agglutination tests using anti-B reagents. Reagents are isoantisera diluted to the point where they will only react with a single isoantigen. The chickens were immunized with washed red cells and the resultant anti-B_2 or anti-B_{14} sera tested against B_1, B_2, B_{13} and B_{14} cells for titre and cross-reactivity. Those birds which produced isoantisera which did not cross react were selected for further immunization.

3. Genotypic Matching

A total of ninety chickens were genotyped by agglutination tests, using reagent antisera, for alleles at the A, B, C, D, E, L and Z loci. From these chickens, donor

tested by agglutination using B_1, B_2, B_{13} and B_{14} washed cells. If it agglutinated any type of cells, other than the type used for immunization, the isoantiserum was adsorbed with cells of the genotype it cross reacted with. A pool of washed cells was prepared by mixing cells from two to four birds. After the final centrifugation all supernatant was removed to avoid dilution of the isoantiserum. The isoantiserum was added to the packed cells and allowed to agglutinate for 2 hours under constant agitation at room temperature. Complete exhaustion of cross reactivity could be obtained without significant loss of strength of the isoantiserum, if large enough numbers of cells were used. As much as 20 ml. of packed cells per ml. of serum were sometimes necessary for complete adsorption. Usually, however, 2 ml. of cells were sufficient to adsorb the cross reactivity from 1 ml. of serum.

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recipient pairs were selected so that the donor possessed a B antigen not present in the recipient and the other loci contributed no foreign antigens. For example:

Donor genotype	$A^6A^6\ B^2B^{14}\ C^1C^5\ D^2D^2\ E^8E^{10}\ L^2L^2\ Z^1Z^1$
Recipient genotype	$A^2A^6\ B^{14}B^{14}\ C^1C^5\ D^1D^2\ E^8E^{10}\ L^2L^2\ Z^1Z^2$
Possible isoantibodies	none anti B_2 none none none none none

The resultant antisera were tested against B_1, B_2, B_{13} and B_{14} cells for titre and cross-reactivity. Chickens which produced non-cross-reacting isoantisera were selected for further isoimmunization.

VI. Mammalian Antisera

All mammalian anti-sera used in this study were purchased from commercial suppliers. When not in use they were stored at -20°C.

Product	Code	Lot #	Supplier
Anti-chicken serum (goat serum)	CS2178	1 2	Colorado Serum Co., Denver, Co.
Anti-chicken globulin (rabbit serum) fluorescein conjugated	6921	2684	Mann Research Labs. Inc. New York, N.Y.
Anti-chicken globulin (rabbit serum)	9181	2695	"
Anti-chicken globulin (rabbit globulin) fluorescein conjugated		1008632	Sylvana Co. Millburn, N. J.
Anti-chicken globulin (rabbit globulin)		221615	"
Anti-chicken globulin (rabbit serum)		0528621	"
Anti-chicken globulin (rabbit serum) fluorescein conjugated		104607	"
Anti-rabbit globulin (horse serum) fluorescein conjugated		0117633	"

VII. Preparation of Specific Fluorescent Isoantibodies

1. Fluorescent Dye-Protein Conjugation

The conjugation procedure followed was essentially that described by Riggs et al. (1958), except that acetone was not added to the reaction mixtures (Cohen et al. 1960). Fluorescein isothiocyanate (FITC) and Rhodamine B 200 (RB 200), as the isothiocyanate, were purchased from Hyland Laboratories, Los Angeles, California. They were supplied in a stable powdered form and could be stored for long periods at room temperature. A refractometer reading was taken to determine protein concentration and the protein solution buffered at pH 9.0 with 0.1 M carbonate buffer, 0.3 ml. per ml. of serum. For each mgm. of protein 0.05 mgm. of FITC or RB 200 was added to the surface of the serum to provide a 1-20 dye/protein ratio. The reaction mixture was placed in an insulated bucket packed with ice and agitated slowly for 6 to 10 hours on a Fisher Clinical Rotator (Fisher Scientific Co.) In order to avoid foaming, a speed of 70 r.p.m. was not exceeded. After conjugation, the labelled serum was refrigerated at 4°C until chromatographed.

2. Serum Fractionation

(a) Ammonium Sulphate Precipitation of Globulins

A saturated solution of ammonium sulphate was prepared by adding 754 gm. of ammonium sulphate to 1 litre of distilled water and allowing it to dissolve for several hours at 20°C.

A 10 ml. sample of serum in a centrifuge tube was placed in an ice bath. To this was added 10 ml. of saturated ammonium sulphate at the rate of 1 ml. per minute while stirring constantly. The solution was refrigerated at 4°C for 3 to 4 hours and the precipitated globulins centrifuged at 2000 r.p.m. in a refrigerated centrifuge. After discarding the supernatant fluid, the precipitate was redissolved in 10 ml. of distilled water by stirring gently and reprecipitated as before. A white precipitate was usually obtained after two precipitations. If the serum was badly hemolyzed a third precipitation was

sometimes necessary to remove hemoglobin.

The final precipitate was dissolved in 8 ml. of distilled water and dialyzed against phosphate buffered saline, pH 7.3, to remove sulphates. The protein solution was poured into a 27/32 inch diameter dialysis bag with a wall thickness of 0.0010 inches (Visking Corporation, Chicago, Ill.) and both ends were securely tied. This was placed in a one litre beaker containing the phosphate buffered dialysate, refrigerated at 4°C, and allowed to dialyse for 24 hours. After a further 8 hours the dialysate was tested for the presence of sulphates. Equal amounts of 1% barium chloride and the dialysate were combined and allowed to stand for 1 hour. If the solution did not become cloudy, the protein solution was considered to be free of sulphates. If a positive (cloudy) reaction was observed, the buffer was changed and tested again when convenient.

When free of sulphates, the concentration of the protein solution was determined using the refractometer and adjusted to between 1.5 and 2.0 gm. % by dilution with phosphate buffered saline, or concentration by vacuum dialysis. Conjugation of the globulins with fluorescent dye was carried out in the same manner as for serum.

(b) Chromatography

(i) Description of Columns

Although suitable chromatographic columns are available from Pharmacia Fine Chemicals, Inc., New York, N.Y., we preferred to make our own. A #4 Pyrex stop cock and a #24/40 ground Pyrex glass joint were fitted to either end of a 2.5 cm. inside diameter glass tube, 25 cm. in length. A 3.5 cm. inside diameter glass tube with inlet and outlet was fitted around the column to provide a cooling jacket. A small amount of glass wool under a 1 cm. thickness of 1/8 inch diameter glass beads was placed in the bottom of each column to support the gel bed.

Two such columns were fabricated; they could be used singly or connected in tandem, using rubber joints made by drilling holes in #5 rubber stoppers.

(ii) Preservatives

Sera and protein solutions were refrigerated at 4°C when not in use, or stored frozen at -20°C. During chromatography, and other procedures, protein solutions were kept at 4°C using ice baths, if the process took longer than a few minutes. Ice water was circulated through the cooling jackets of the chromatographic columns while fractionation was in progress. An immersible pump (Pump #1, Little Giant Pump Co., Oklahoma City, Okla.) provided a constant flow of coolant.

Sodium azide was used as a preservative for conjugated dye-protein solutions in a concentration of 1 : 10,000 W/V. It was also included in all buffer solutions except 0.1M carbonate buffer, pH 9.0, used in the conjugation procedure.

(iii) Preparation of Chromatographic Adsorbents

DEAE

N, N - diethyl aminoethyl ether (DEAE) cellulose was purchased from Eastman Organic Chemicals, Rochester, N.Y. Before use the cellulose powder was prepared in 20 gm. batches following precisely the method of Peterson and Sober (1962) and suspended in the starting buffer (0.02M phosphate buffer pH 6.0) in a ratio of 1 gm. of DEAE per 60 ml. of buffer.

Sephadex

Sephadex G-25 and G-200 were supplied in bead form by Pharmacia Fine Chemicals, New York. Sephadex must be completely hydrated before packing in a column or the gel will continue to swell in the column. It requires no washing. A 10 gm. batch of G-25 or a 20 gm. batch of G-200 could be prepared and stored in the

refrigerator until use. The Sephadex beads were poured into a large excess of .01 M phosphate buffered saline pH 7.3 and allowed to hydrate for 24 hours and settle. The supernatent was suctioned off to remove fine particles and the Sephadex resuspended in buffer and stored.

(iv) Packing the Columns

The packing procedure was essentially the same for DEAE, Sephadex G-25 and Sephadex G-200. A 1 litre separatory funnel was attached to the top of the column with a rubber joint to provide a reservoir. An electric stirrer was mounted as in Figure 1. The adsorbent suspension was poured into the reservoir and the stirrer started. About 50ml of the suspension was allowed to enter the column and settle out under gravity to a thickness of 2 cm. This prevented the adsorbent from flowing through the glass wool and beads. By opening the upper and lower stop cocks and releasing the air pressure inside, the column could now be filled with the suspension and allowed to run freely. A steadily rising, level bed of adsorbent was maintained, without stopping, until the desired height was achieved. Finally, a filter paper disc was allowed to settle onto the top of the bed for protection when applying samples. Approximately 3 hours were required to pack a column with a bed volume of 100 ml

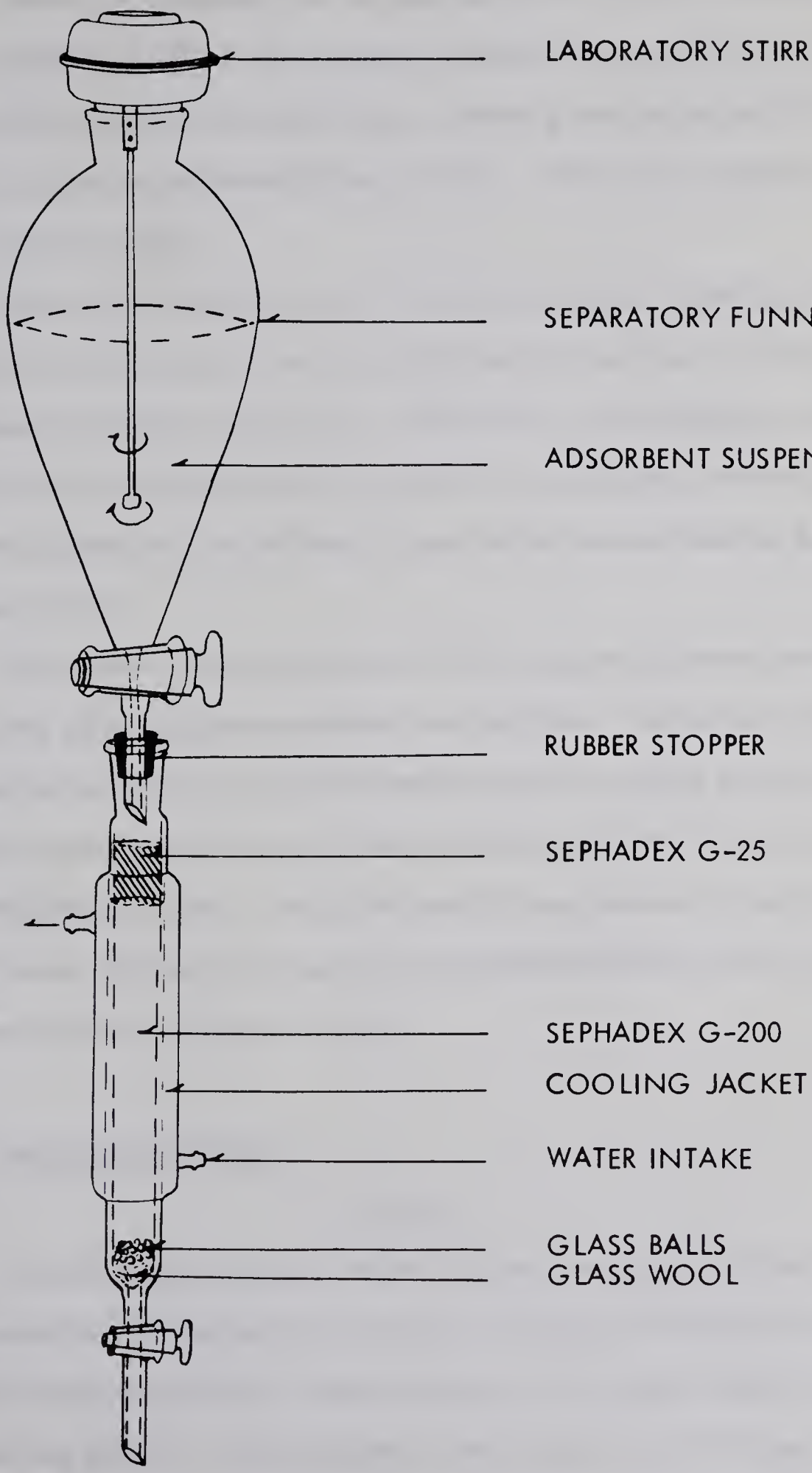
Sephadex columns required no further packing; however, DEAE columns were compressed with air pressure to provide a more physically stable bed. An adjustable air pressure valve was attached to the top of the reservoir which contained starting buffer only. The column was allowed to run and the pressure gradually increased until 10 lbs. p.s.i. had been reached, taking care that the buffer level did not go below the top of the DEAE bed.

The progress of the fluorescent proteins down the column was followed by observing their fluorescence under long wave ultraviolet illumination provided by a

Blak Ray B-100A Lamp (Ultra Violet Products Inc., San Gabriel, Cal.) Following the appearance of the first fluorescent drop at the outlet, the effluent was collected in test tubes in an ice bath. Separation of the protein and free dye could be clearly observed using the ultra-violet lamp. When all the protein was collected, the dye and buffer salts were washed out of the column with distilled water and the column re-equilibrated.

In later stages of these experiments, 1 ml. serum samples were used. It was found that Sephadex G-25 could be packed directly on top of Sephadex G-200 in the same column. This enabled us to remove free dye, change buffer salts and fractionate the serum in one operation.

CHROMATOGRAPHIC APPARATUS



SCALE 1 : 4

Figure 1.

(v) Preparation of Protein Samples

Before the fluorescent dye conjugated protein could be fractionated on DEAE or Sephadex G-200 it was necessary to equilibrate the proteins with the starting buffer and remove excess free dye. Initially this was accomplished using a separate column packed with Sephadex G-25. A 50 ml. bed volume was used to prepare a 10 ml. sample.

If the column had been used it was regenerated by attaching a 20 litre reservoir of distilled water to the inlet and allowing it to flow for 72 hours or until all fluorescence had been washed out. Equilibration of the Sephadex with phosphate buffered saline at the desired molarity and pH was achieved by running buffer through the column until the effluent pH was that of the equilibrating buffer. This took about 6 hours.

The column was shut off and all buffer above the Sephadex bed removed by suction using a Pastuer pipette attached to a trap flask. The protein sample was then pipetted carefully onto the filter paper so as not to disturb the top of the gel. The column was allowed to run until the sample had sunk into the gel just to the level of the top of the bed. The buffer reservoir was reconnected and the column allowed to run. Caution was required to assure that the liquid level in the column was never lower than the top of the gel.

VIII. Concentration Methods

1. Osmosis

Flakes of high molecular weight polyethylene glycol (Carbowax 20,000, Fisher Scientific) were ground with a mortar and pestle and funneled into a length of 27/32 inch dialysis tubing, closed at one end. The protein solution to be concentrated was placed in a glass cylinder a little wider than the tubing. The tubing containing the Carbowax was immersed in the solution and refrigerated so that

water and salts entered the tubing, concentrating the protein solution. (Lipp, 1961).

2. Vacuum Dialysis

The apparatus in Figure 2b was purchased from Schleicher and Schuell Co., Keene N.H. A sample of protein solution was placed inside the collodion tube and the outer chamber filled with distilled water to within 1 cm. of the top of the bag. The apparatus was placed in the refrigerator and the outer chamber connected to a vacuum pump by a line passing through the refrigerator wall. Vacuum pressure was adjusted to 20 cm. of mercury using a bleeder valve. Higher pressure caused the joint at the top of the bag to leak. Concentration rate decreased from 4-6 ml. per hour to 0.25 - 0.5 ml. per hour as concentration proceeded.

The six bag ultrafiltration apparatus in Figure 2a is a modified version of that described by Peterson and Sober (1962). The reservoirs are 50 ml. drying tubes (Fisher Scientific Co.,) whose orifices fit snugly into 3/8 inch dialysis tubing (Visking Corp., Chicago, Ill.). Number 5 rubber stoppers with 1/4 inch holes drilled through them will slip over the tubing when wet and provide an air tight seal when vacuum pressure is applied, due to their tapered shape. The top is an 11 inch disc of lucite, 3/4 inches thick, which is sealed to the glass dessicator with stop-cock grease.

Each reservoir has a 75 ml. capacity and when run at a vacuum pressure of 20 cm. of mercury the concentration rate decreased from 4-6 ml. per hour to 0.25 - 0.5 ml. per hour with increasing concentration.

Serum samples could be poured into each reservoir but were removed after concentration with a catheter attached to a syringe. To wash the bags after use, distilled water was poured into each reservoir and removed by catheter from the bottom of the bag.

VACUUM CONCENTRATION APPARATUS

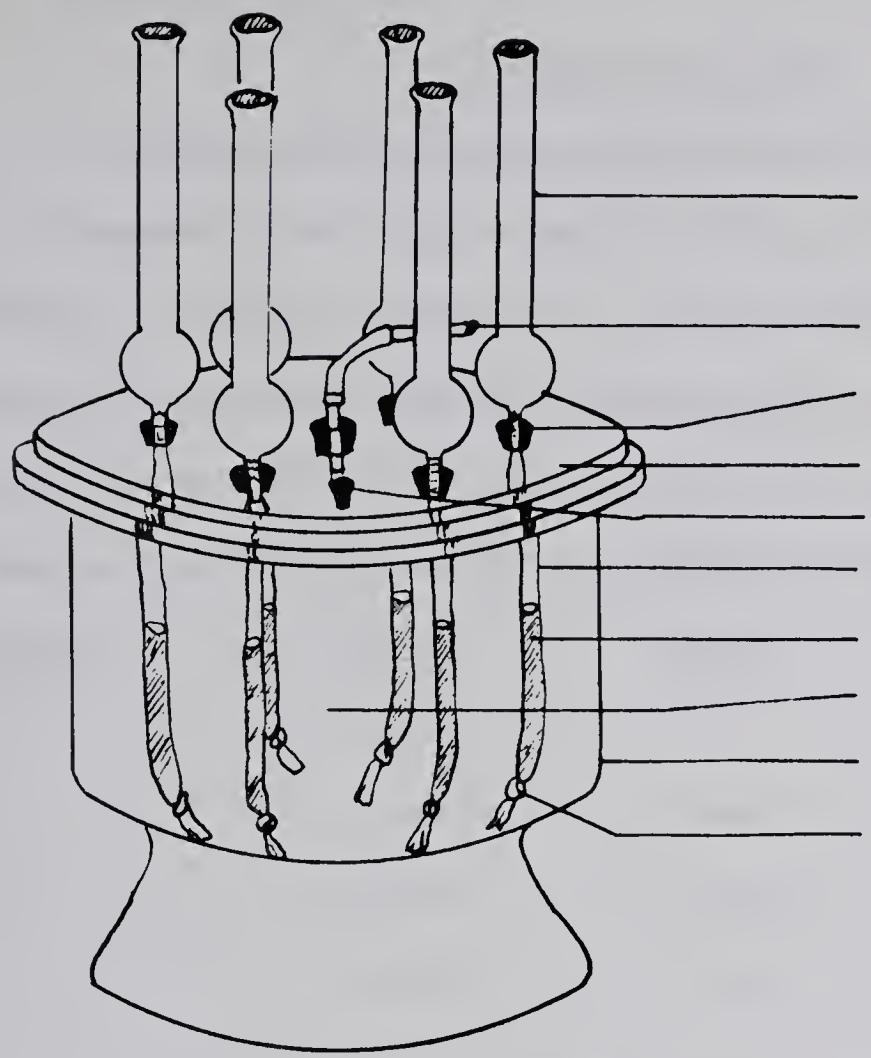


Figure #2a.

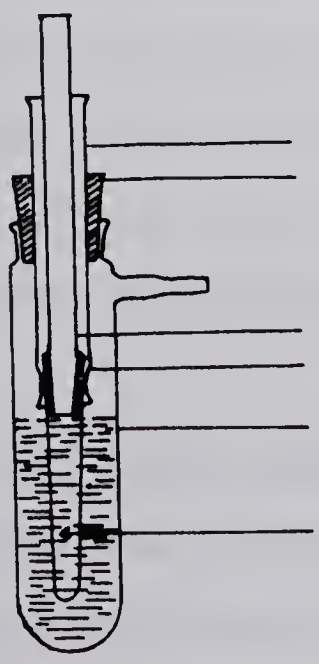


Figure #2b.

IX. Bioassay Methods

1. Agglutination Tests

Agglutination tests were performed using a Microtiter apparatus (Cooke Eng. Co.; Alexandria, Va.) Single drops of a 2% suspension of red cells were added to 0.025 ml., of serum in serial dilution. Resultant titres were read macroscopically, a button of sedimented cells constituting a negative result. A positive well was one in which at least 75% of the cells remained on the surface of the conical bottom. Titres were calculated according to total dilution of antiserum. For example:

Well No.	Serum	Saline	Cell suspension	Titre
1	1 part		1 part	1 : 2
2	1 part	1 part	1 part	1 : 3
3	1 part	3 parts	2 parts	1 : 6
4	1 part	7 parts	4 parts	1 : 12

2. Immune Electrophoresis

Immune electrophoresis analyses were carried out using LKB 6800A micro apparatus (LKB Produkter AB, Stockholm, Sweden) in a supporting medium of agar gel on microscope slides, according to directions in LKB manual #16800-AE_{01} based on the theory described by Hirschfeld (1960). Goat serum (anti chicken serum) was the antiserum used in all cases, and the antigens were chicken serum or fractions of chicken serum. The staining was prolonged to 24 hours to bring out faint lines.

3. Cellulose Acetate Electrophoresis

Electrophoresis of serum and serum fractions was carried out using a Beckman model R-101 microzone electrophoresis cell (Beckman Instruments). Separation of serum components was performed on cellulose acetate membranes in barbital buffer at pH 8.6 according to instructions in Beckman Technical Bulletin # RM-1M-2

(1963). Relative concentration of the fractions was charted by making densitometer graphs of the cleared membranes carrying stained bands of protein. A Beckman Model R-102 Microzone Scanning Attachment was used.

X. Preparation of Test Materials

1. Frozen Tissues

(a) Quick Freezing

(i) Whole embryos

Small chunks of dry ice were dropped into acetone in a 500 ml. Pyrex beaker. A 200 ml. stainless steel beaker containing isopentane was suspended in the acetone. More dry ice was added until the temperature of the isopentane had dropped to -65°C. A fresh, whole embryo plunged into the isopentane could be frozen in a few seconds.

An alternative method, using liquid nitrogen in an open-mouthed Dewar flask as the coolant, was used. The isopentane temperature dropped to -120°C at which point the isopentane began to freeze. The steel beaker was removed from the nitrogen and the embryo plunged into the isopentane to freeze almost instantly. The embryo was removed to a pre-cooled container and put in the freezer at -20°C.

(ii) Organs

Organs from mature chickens or embryos are usually inconvenient for frozen sectioning without being mounted in a supporting block. O.C.T. (Lab-Tech, Westmount, Ill.) is a liquid gelatin which freezes to a resiliant solid at 0°C and is alcohol soluble. A drop or two of O.C.T. was poured into a number 100 gelatin capsule and the organ or piece of tissue suspended in it. The capsule was filled with O.C.T. and all air bubbles removed by tilting gently back and forth. The gelatin cap was put in place and the capsule labelled with a masking tape label. The capsule was then quick frozen in isopentane as usual and stored at -20°C.

(b) Frozen Sectioning

Sections were cut in a Harris-International cryostat (Harris Refrigeration Co., Cambridge, Mass.) on a rotary microtome (International Equipment Co., Boston, Mass.) at a temperature of -20°C. Quick frozen material was stored for at least 12 hours in the cabinet so that it would come up to temperature. Embryos or O.C.T. blocks were mounted on the microtome mounting discs using ice as the adhesive. The discs were placed in a drilled wooden block so that the mounting surface was horizontal. A mound of ice was built up by applying drops of water to the surface of the disc with a pipette. When the surface of the ice mound was just freezing the embryo or O.C.T. block was applied and frozen into place, without melting. Additional ice could be built up around the material for added stability by applying successive small amounts of water.

The gelatine capsules were peeled off the O.C.T. at this point, as they are too rigid to section.

The mounting discs were clamped in the microtome and sections cut at 3 micra. Camel hair brushes were held in each hand and used to keep the sections from curling, and to transfer the sections to a pre-cooled slide, as each cut was made. When the sections had been satisfactorily flattened on the slide, they were melted to it by pressing the underside with the thumb. The slides were removed from the cryostat and air dried. No other adhesive was necessary.

2. Cell Suspensions

Erythrocytes and leukocytes were prepared for fluorescent labelling by washing with Alsever's solution. A 0.5 ml. sample of citrated blood was centrifuged in a disposable plastic centrifuge tube, in a Beckman Microcentrifuge (Model 152, Beckman Instruments) for 1 minute. The plasma was pipetted off and the cells washed three times in Alsever's solution. After the final centrifugation they were

diluted to approximately 5% by adding 1 drop (0.025 ml.) of loosely packed cells to 0.4 ml.. of Alsever's solution.

3. Imprints

Mature organs were prepared for imprinting by slicing the tissue with a razor blade so as to provide a flat surface. Imprints were made by pressing the surface gently against a glass slide and air drying at room temperature.

XI. Treatment of Test Materials

1. Fixation and Direct Application of Fluorescent Isoantisera

Sections prepared from frozen material were either fixed in cold 95% ethanol for 20 minutes, suspended in formalin vapour for 30 minutes or used without fixation. After ethanol fixation the sections were brought to water by rinsing gently in 50% ethanol for 10 minutes followed by 10 minutes in phosphate buffered saline. Excess buffer was blotted off and a drop of fluorescent isoantisera applied to the moist sections. Each slide was placed in a separate Petri dish containing a filter paper wet with buffer. The slides in the Petri dishes were agitated gently for 30 minutes on the clinical rotator and then washed for 30 minutes in several changes of buffer solution, in a Coplin jar on the rotator. A cover glass was applied to the wet sections with buffer serving as the mounting medium. Slides prepared in this manner could be examined immediately or stored overnight in the refrigerator in Petri dishes. The procedure used for formalin fixed sections was the same except that they were given only a quick dip in buffer before application of antisera. Imprints were treated in the same manner as sections.

Cell suspensions were always unfixed and handled as wet preparations throughout. The labelling with fluorescent isoantisera was a modified version of the procedure described by Cohen et al. (1960). To three drops of a 5% suspension of

cells, in a polyethylene microtube, was added three drops of the fraction of fluorescent isoantisera being used. The suspension was mixed by tilting and agitation for one hour on the rotator. The cells were centrifuged and washed three times in 0.4 ml. volumes of Alsever's solution. Agglutinated clumps of cells were broken up by flicking the tube sharply with a forefinger. Three drops of Alsever's solution were added after the final wash and the cells mixed. One drop of the suspension was placed on a slide and cover glass applied. The slides were kept from drying by storage in a Petri dish, as for sections.

2. Indirect Technique

The multiple layer or "sandwich" technique was employed to enhance weak fluorescence of blood cells in suspension (Jankovic', 1959). Cell suspensions that had been incubated with fluorescent isoantibodies were washed and incubated for one hour with fluorescent rabbit or goat antibodies (anti-chicken globulin). In one of the early experiments the fluorescence was enhanced by a further incubation with fluorescent horse antibodies (anti-rabbit globulin).

XII. Comparative Methods

1. Acridine Orange Staining

Frozen tissue sections, smears of cell suspensions and organ imprints were fixed in ether:ethanol, and stained with acridine orange according to the method of von Bertalanffy (1956). Examination under ultra-violet light of the bright red and green fluorescence associated with RNA and DNA provided a method of comparing cell types and morphology with similar preparations that had been labelled with fluorescent antisera.

All materials were handled as wet preparations after fixation and were examined, using the aqueous buffer as a mounting medium. Where a permanent

record was desired the materials were photographed, as the fluorescence tends to fade rather quickly.

2. Toluidine Blue

Where a permanent mount of a section or other preparation was required, and as an alternative method of viewing test materials using transmitted tungsten light, preparations were stained with Toluidine Blue. Sections or cells were air dried at room temperature and fixed in 50% ether: 50% ethanol for 5 minutes. They were then passed quickly through 80%, 70%, 50% ethanol solutions into distilled water. Sufficient staining occurred in 2 minutes in a 0.2% solution of Toluidine Blue at pH 7.4. This was followed by rinsing in distilled water, 50%, 70%, and 95% ethanol. Finally, the slides were passed through two xylene baths and mounted in Canada Balsam.

3. Phase Microscopy

The Leitz phase microscope was used to view and photograph cells in suspension which had been labelled with fluorescent antisera and did, or did not, show fluorescence. By leaving a slide in place on the microscope stage and changing optics it was possible to view the same cells by phase contrast and under ultra-violet excitation.

XIII. Microscopy

1. Description of Microscopes

(a) Reichert

Preliminary fluorescence microscopy was done using a Reichert "Zetopan" microscope.

The ultra-violet light source was a 200 watt, high pressure, mercury

vapour arc lamp (Osram HBO 200), mounted in a lamp housing equipped with an adjustable mirror and condenser lens, a field diaphragm and permanent heat absorbing filter.

An E2 Reichert filter (glass type/thickness: UG1/1.5 mm.) and a quartz cell containing a solution of 2% copper sulphate were the exciter filters used. An SPZ (GG13/1 + 3 mm. + Wratten Foil 2B) U.V. adsorbing filter or SP3 (GG9/1 mm. + OG 1/1.5 mm.) U.V. - Blue adsorbing filters were the barrier filters employed.

These filters were used in conjunction with an Abbé type three lens condenser, made from ultra-violet transmitting glass and requiring immersion oil between the top lens and the microscope slide. For low power viewing Reichert dry achromatic objectives were adequate. For high power a Zeiss "Neoflar" 100x objective was used.

Specimens were photographed with a Remica II 35 mm. camera (Reichert) mounted on the standard camera tube which is equipped with a fixed beam-splitter prism and focusing telescope, a camera shutter and cable release.

(b) Leitz

A Leitz Ortholux microscope equipped with optics for ultra-violet, phase, and transmitted light microscopy was used in the latter stages of the work.

Ultra-violet illumination consisted of two HBO 200 lamps in separate housings to provide both transmitted and incident excitation of fluorescent specimens. The exciter filters on each lamp were the same: glass type/thickness: UG5/5 mm. and BG 38/5 mm. (comparable to a copper sulphate cell). The barrier filters employed above the microscope stage were Leitz U.V. suppression filters screwed into the eyepieces and an ultra-violet suppression and blue absorbing filter in the camera tube light path.

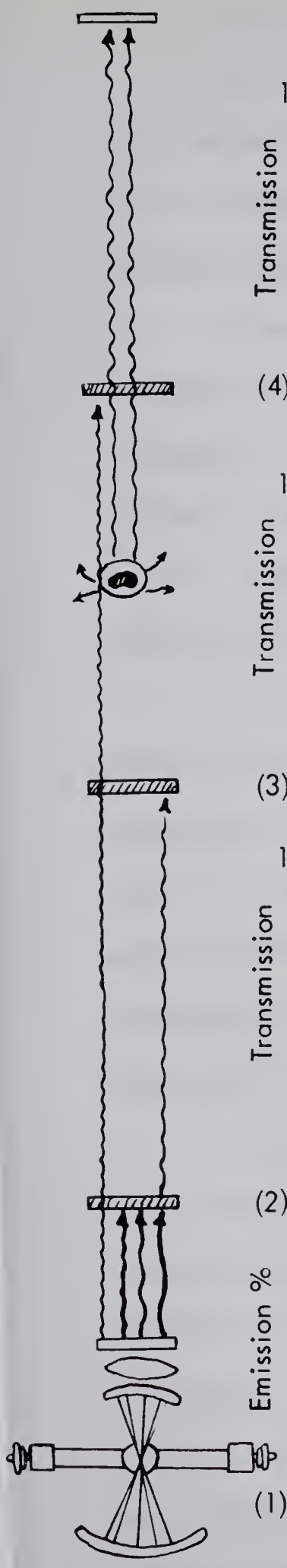
A three lens bright field condenser (1.40 N.A.) focused the transmitted light. The "Ultropak" incident objectives each have a built in condenser to focus incident illumination. Ultropak objectives of 3.8, 11, 32 and 100 power were used. All lenses and filters are made of special ultra-violet transmitting glass.

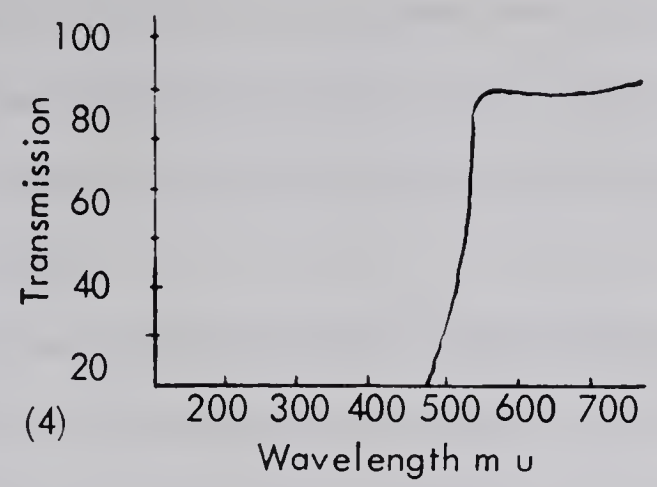
For phase contrast microscopy the objectives and condenser were replaced with a Leitz variable phase contrast condenser and 10, 45 and 90 power apochromatic phase objectives. A tungsten light source was used for illumination.

For examination of stained material the tungsten light source was employed with a two diaphragm bright field condenser and Leitz plan objectives.

The photographic equipment included a Reichert Remica II 35 mm. camera and a Leica 35mm.camera with focal plane shutter and screw base, mounted on a camera tube equipped with a swing out beam-splitter prism coupled to the camera shutter and a focusing telescope.

CHARACTERISTICS OF EXCITER AND ABSORBING FILTERS

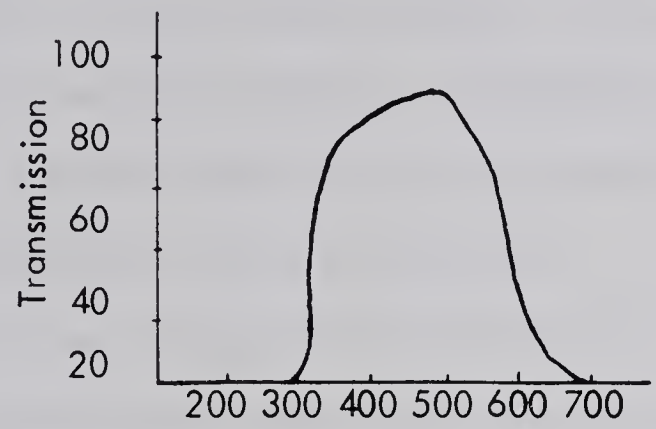




(4)

ULTRA-VIOLET-BLUE ABSORBING FILTER (4)

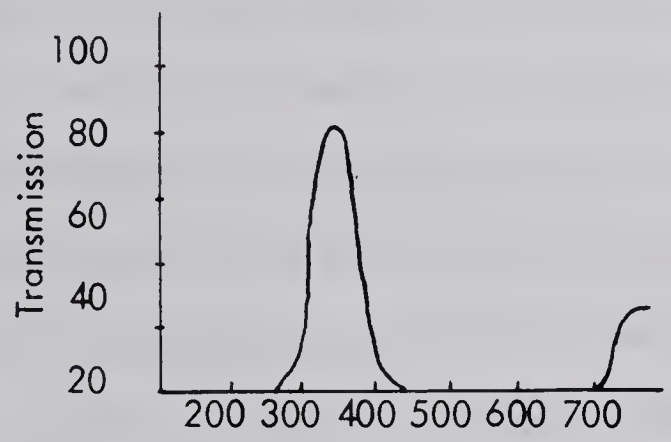
Reichert	SP 3
Leitz	U.V.-BLUE ABSORBING



(3)

RED ADSORBING (3)

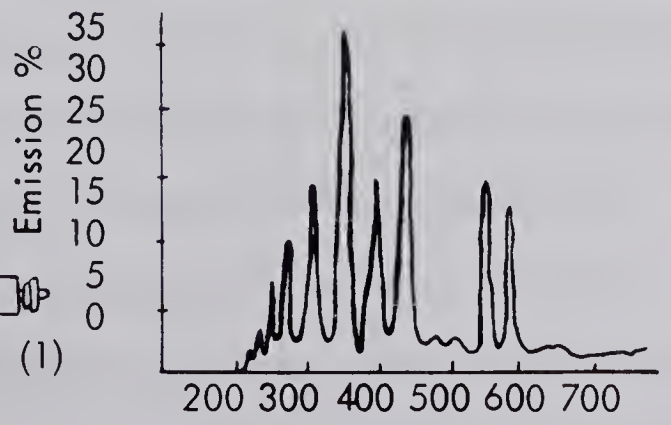
Reichert	$CuSO_4$
Leitz	BG 38



(2)

EXCITER FILTERS (2)

Reichert	E 2
Leitz	UG 5



(1)

LIGHT SOURCE (1)

Reichert	OSRAM HBO 200 MERCURY VAPOUR ARC LAMP
Leitz	OSRAM HBO 200 MERCURY VAPOUR ARC LAMP

Figure #3.

XIV. Microphotography

Black and white photographs were taken, on the Leitz Ortholux, of specimens under transmitted light and phase contrast using Kodak Panatomic X (ASA 40) 35 mm. film. For black and white photographs of fluorescence Kodak Tri-X 35 mm. film was used (ASA 200).

A model 501 M photovolt meter (Photovolt Corp.) was used to measure light intensity through the focusing telescope. Test films were exposed using Köehler illumination and constant light intensity. The focusing prism was swung out of the light path during each exposure so that 100% of the available light imprinted on the film. Exposure times were doubled successively. The films were developed and printed according to standard procedures.

Colour photographs of fluorescence or phase contrast were taken using Kodak High Speed Ektachrome colour transparency film (ASA 120). Test films were exposed in the same manner as for black and white photographs except that light meter readings were used only as a guide to the general exposure range. The overall conditions of colour photography (type of fluorescence, filters, condenser and objective) were standardized and three exposures taken of each specimen; for example, 1 second, 2 seconds and 4 seconds.

The colour transparencies were developed by a commercial colour laboratory under standard conditions. Colour negatives were made by contract printing the transparencies using Kodacolor X film (ASA 64). A C-22 Kodak developing kit was used to develop the negatives using varying developing times for each negative, depending on the quality of the transparency. Sodium hydroxide (2.5 gm./litre) was added to the developer to increase its activity and enhance contrast. The negatives were printed on Agafacolor paper using standard processing chemicals with an increase of developing time from 5 to 7 minutes, to further increase contrast.

RESULTS

1. Skin Grafts

Our first problem was to establish the role of the B locus in transplantations within our stock. Although B isoantigens could be recognized by agglutination tests, we thought it necessary to prove that our B isoantigens are transplantation isoantigens. We use skin grafts to show that our B isoantigens are transplantation isoantigens. The first observation was that if mature skin was grafted to chicks that were two weeks old, or older, it was acutely rejected whether it came from a chicken having a foreign B isoantigen or not. It seemed that even minor isoantigenic differences between donor and host caused the sensitive rejection process to react so violently that distinctions could not be made between skin grafts which contained weak from those which contained strong isoantigens. It was assumed that young chicks, in which the immune system was developing, would react less strongly to foreign isoantigens, retain skin grafts longer and provide a less sensitive system for making isoantigenic comparisons.

Accordingly, B^2B^{14} skin was grafted to B^2B^{14} chicks which were seven days old. The grafts healed and then were slowly rejected in varying degrees of intensity. When B^1B^{14}, B^1B^{13} or B^2B^{13} skin was grafted to B^2B^{14} chicks the grafts healed and the rejection was slower than with two week old chicks but clear distinctions between the genotypes of such grafts could not be made. They could, however, be distinguished from B^2B^{14} grafts as they were rejected more quickly.

The next step was to graft B^2B^{14}, B^1B^{14}, B^2B^{13} and B^1B^{13} skin to two day B^2B^{14} chicks. These grafts healed uniformly for the first three days and then became discoloured. B^2B^{14} grafts were retained longer than B^1B^{14} grafts. B^1B^{13} and B^2B^{13} grafts were rejected most quickly. Thus, by grafting mature skin to two day chicks a situation was created in which the rejection process proceeded slowly with the development of the host chicks and comparisons could be made between the effects of B isoantigens.

However, the length of retention of a graft may depend on several physical factors independent of genotype. A much better way of recognizing the genotype of a graft is by its appearance as rejection proceeds. In order to quantitate the relationship between graft genotype, age of grafts and appearance, the following experiments were done. A total of eighty-seven two day old B^2B^{14} chicks were separated into four groups and grafted with B^2B^{14}, B^1B^{14}, B^2B^{13}, and B^1B^{13} mature skin, in two experiments. On the seventh day after grafting, differences between the grafts associated with donor genotype could be seen in some cases. In the interval between the fourteenth and twentieth days the grafts could be divided into three phenotypic groups on the basis of graft appearance only:

Foreign B Isoantigen	Graft genotype	Appearance
B_o	B^2B^{14}	White or pink
B_1	B^1B^{14}	White with brown scabrous patches
B_{13} or $B_1 + B_{13}$	B^2B^{13}) B^1B^{13})	Black or brown scabs

B^2B^{13} and B^1B^{13} grafts were indistinguishable. Table 1 and figure 4 show the accuracy with which the genotype of grafts could be recognized.

On the grafts read on day 6 and day 7, fifty-one out of eighty-seven were guessed correctly, or 58.5%. On days 14, 18 and 19, 92% of the graft phenotypes could be read correctly. By reading, we mean that the genotype of the graft was judged by the appearance of the graft. The 18 and 19 day readings on lot 2 were independently done by different people.

Photographs 1 - 12 are included to illustrate the differences between graft appearance associated with age of the graft and genotype. In the first series of four grafts, at six days after grafting, B^2B^{14} and B^1B^{14} grafts were indistinguishable. They could be distinguished from B^2B^{13} or B^1B^{13} grafts in most cases. The second

series, at nineteen days after grafting, shows more clearly the differences observed between B^2B^{14}, B^1B^{14} and B^2B^{13} plus B^1B^{13} grafts. The black and white photographs serve to illustrate the same point.

Mature chickens were used as skin donors so that several grafts could be taken from a chicken of one genotype and the isoantigenicity of this skin tested on several chicks. Young chicks were used as recipients because of limited animal space and because chicks two weeks old, or older, rejected skin grafts within a few days regardless of the genotype of the graft. It would perhaps have been possible to reverse the conditions and apply skin from younger donors to mature recipients and achieve similar effects, but this was not possible due to technical difficulties.

The data and the photographs demonstrate a marked effect of B isoantigens on the rejection of skin grafts within our stock of genotyped chickens.

SKIN GRAFT RECOGNITION

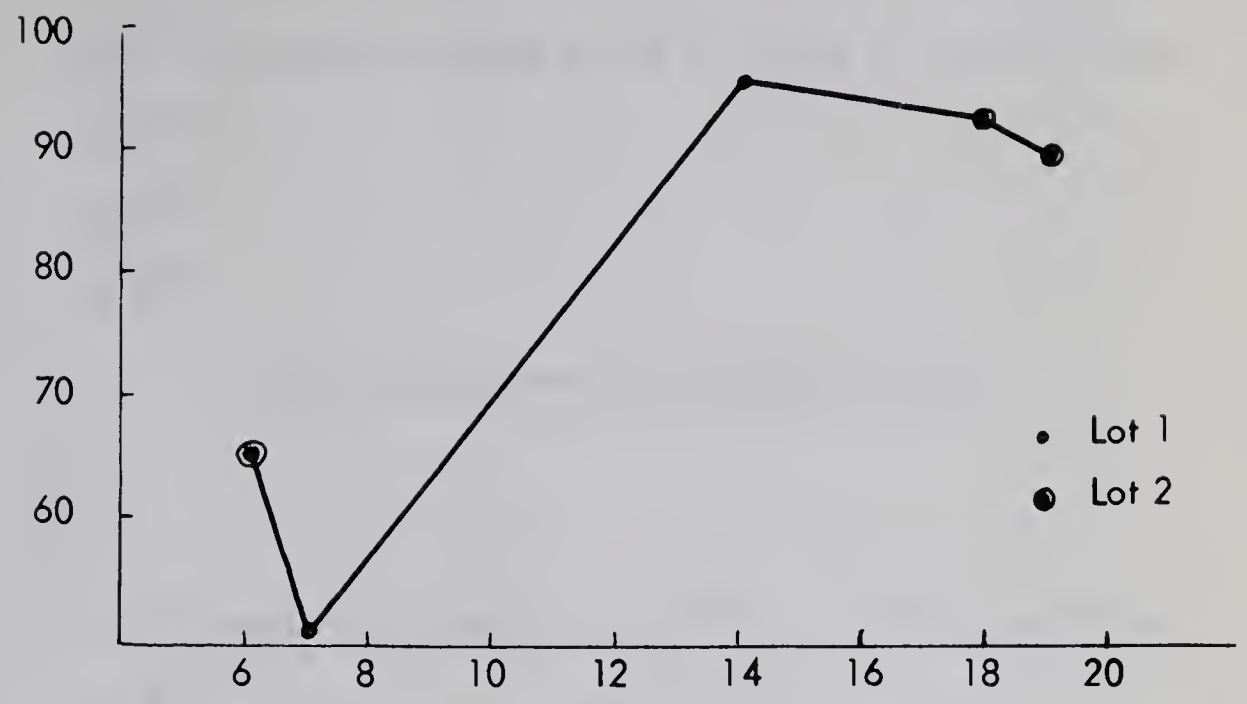


Figure 4. Age of Graft when read, in days.

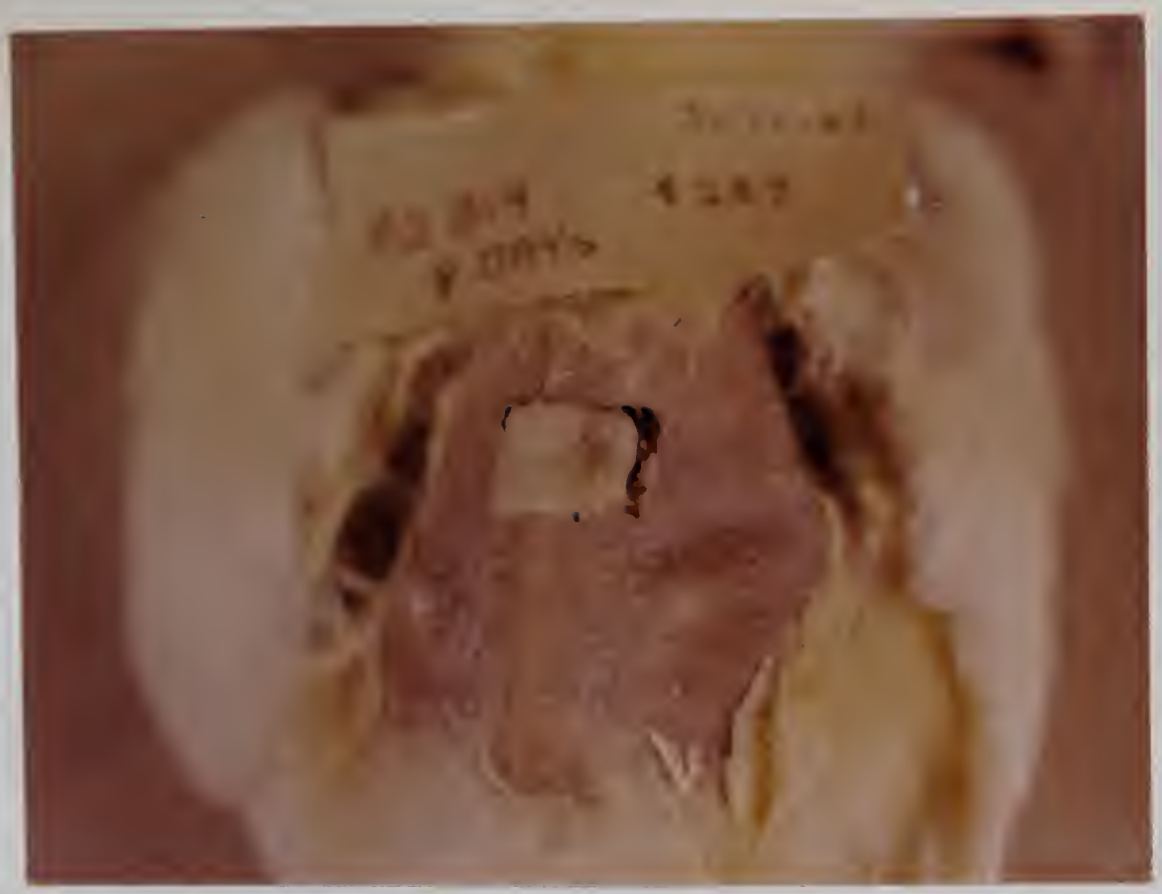
TABLE 1

SKIN GRAFT RECOGNITION

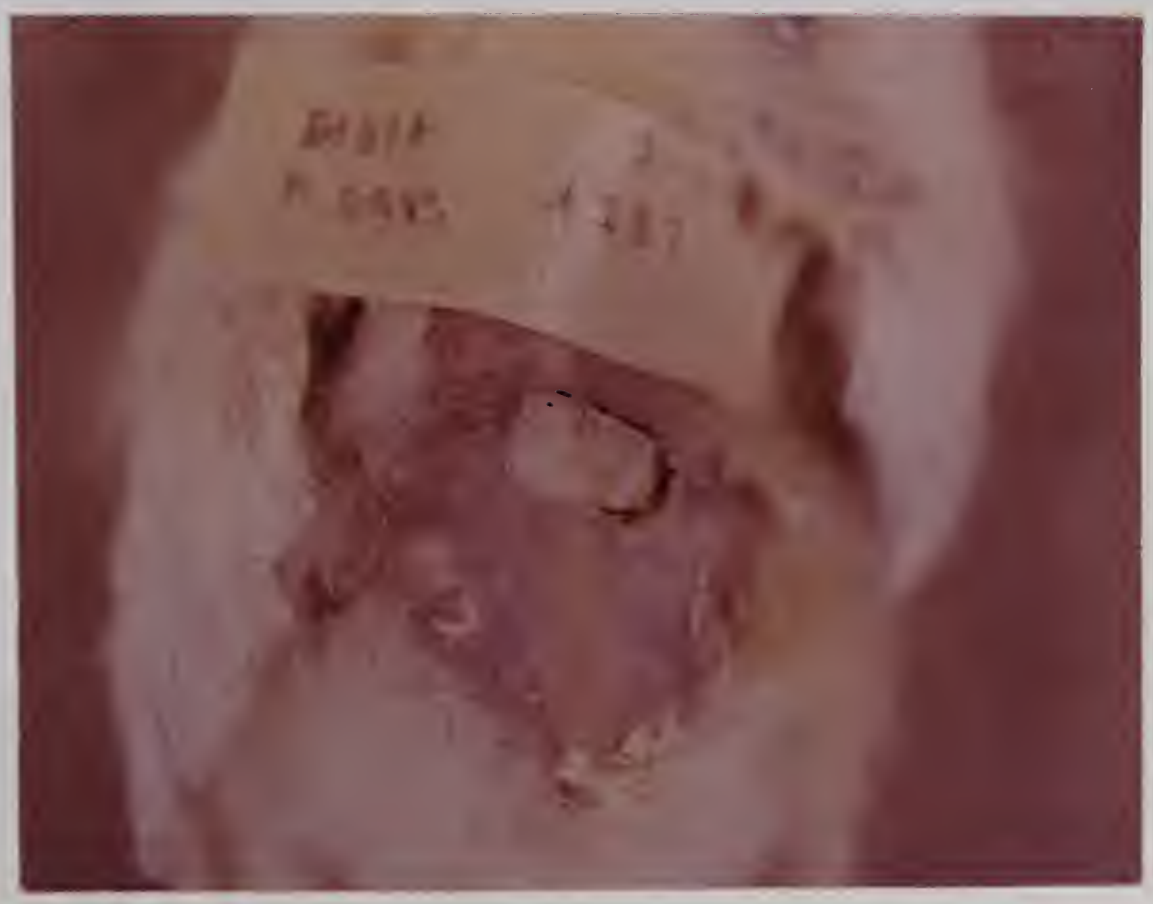
Age of graft when read	B_2B_{14}	B_1B_{14}	(B_2B_{13} & B_1B_{13})	% correct Summation	correctly read / incorrectly read
Lot 1:					
7 days	6/11	7/11	8/19	51.0	21/20
14 days	11/11	10/10	17/19	95.0	38/2
Lot 2:					
6 days	9/11	6/11	15/24	65.0	30/16
18 days	11/11	8/11	16/16*	92.0	35/3
19 days	11/11	8/11	15/16	89.5	34/4

* 8 birds died between the 6th and 18th days

SKIN GRAFTS

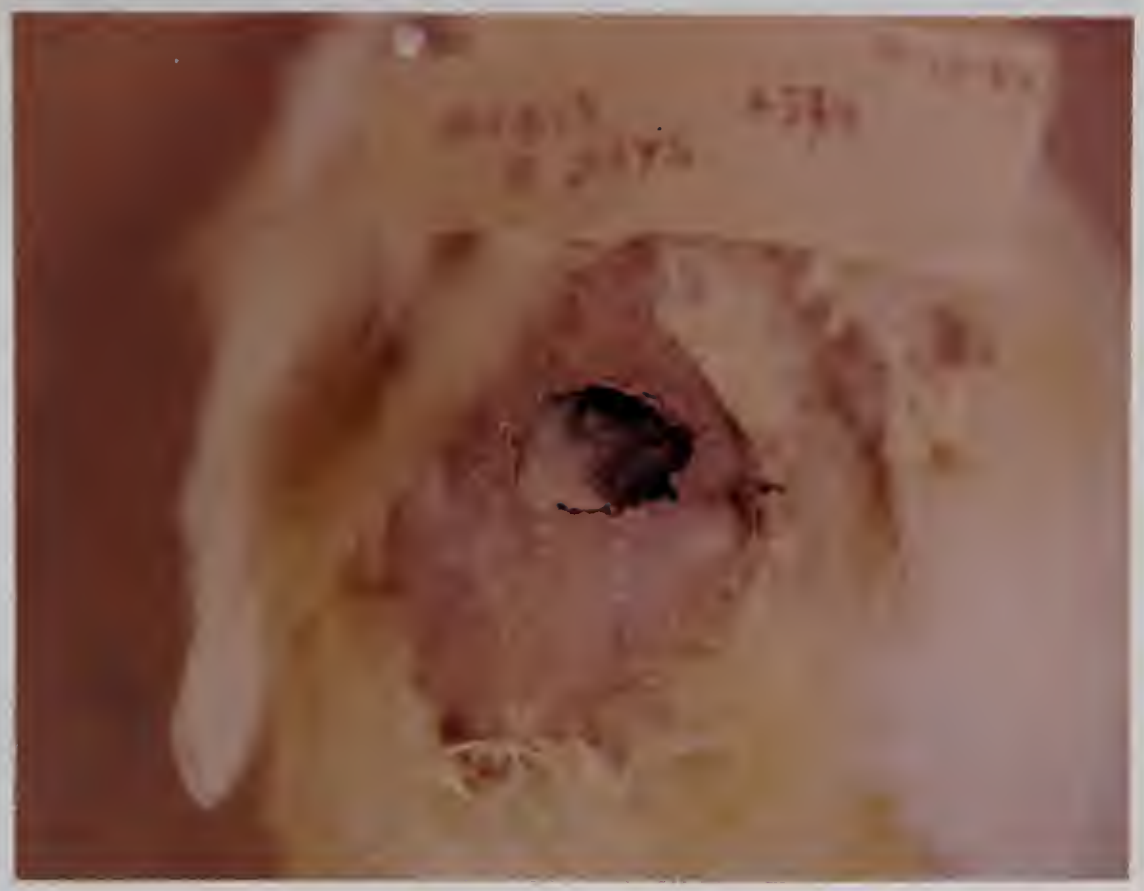


Photograph #1



Photograph #2

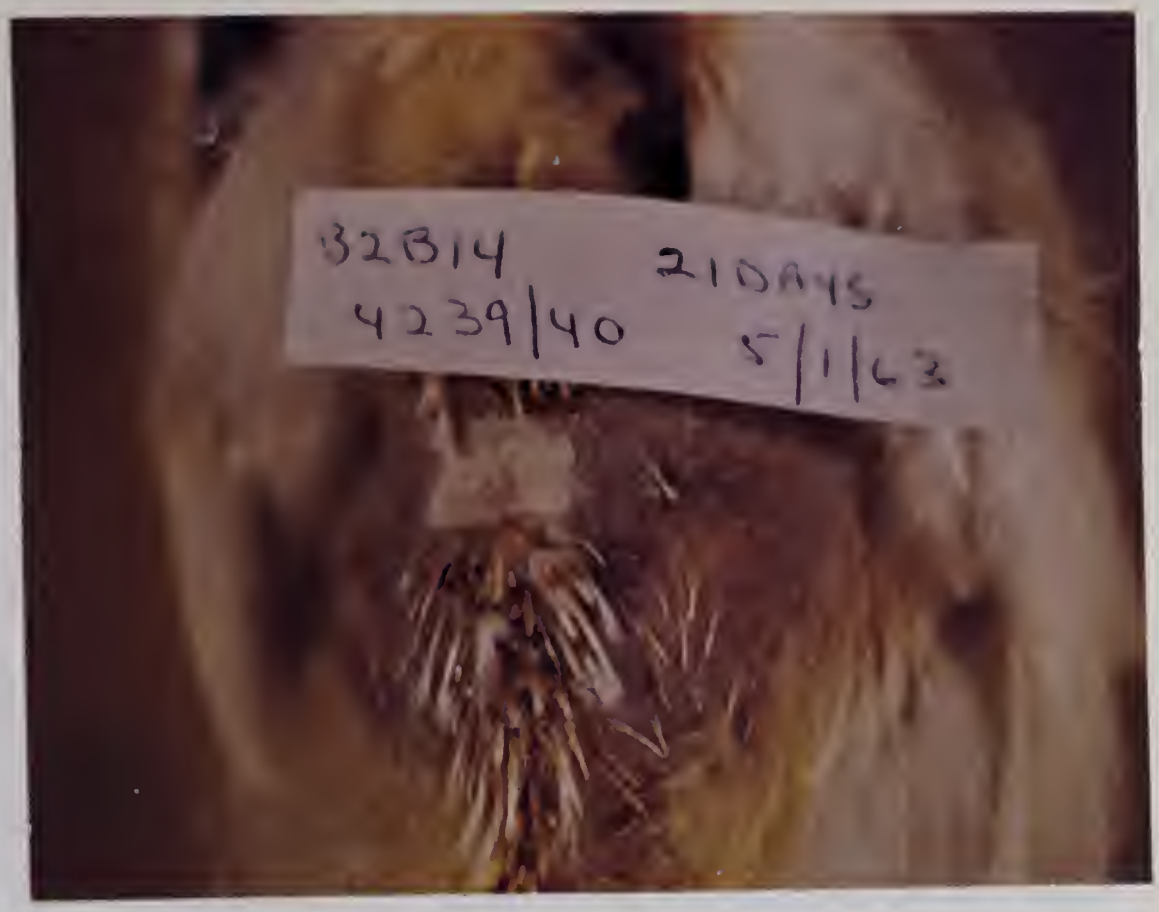
SKIN GRAFTS



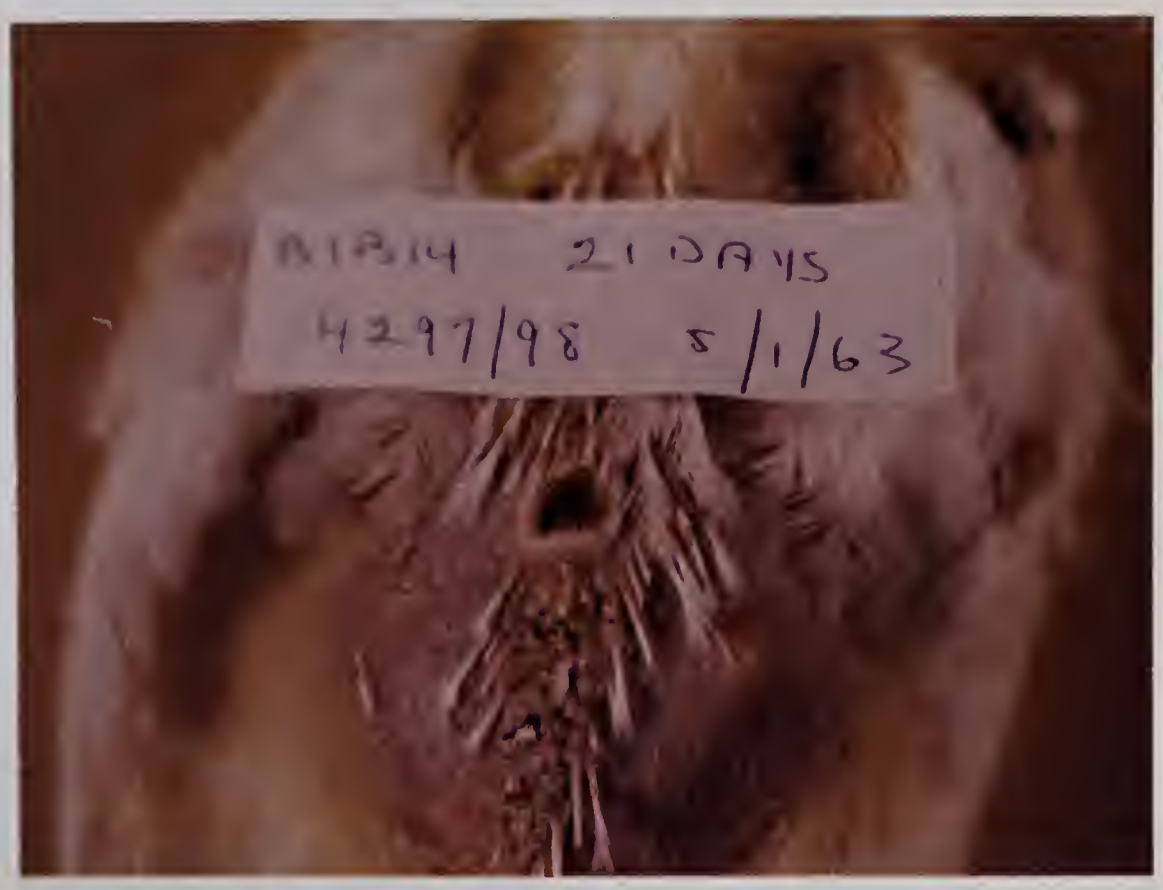
Photograph #3

Photograph #4

SKIN GRAFTS

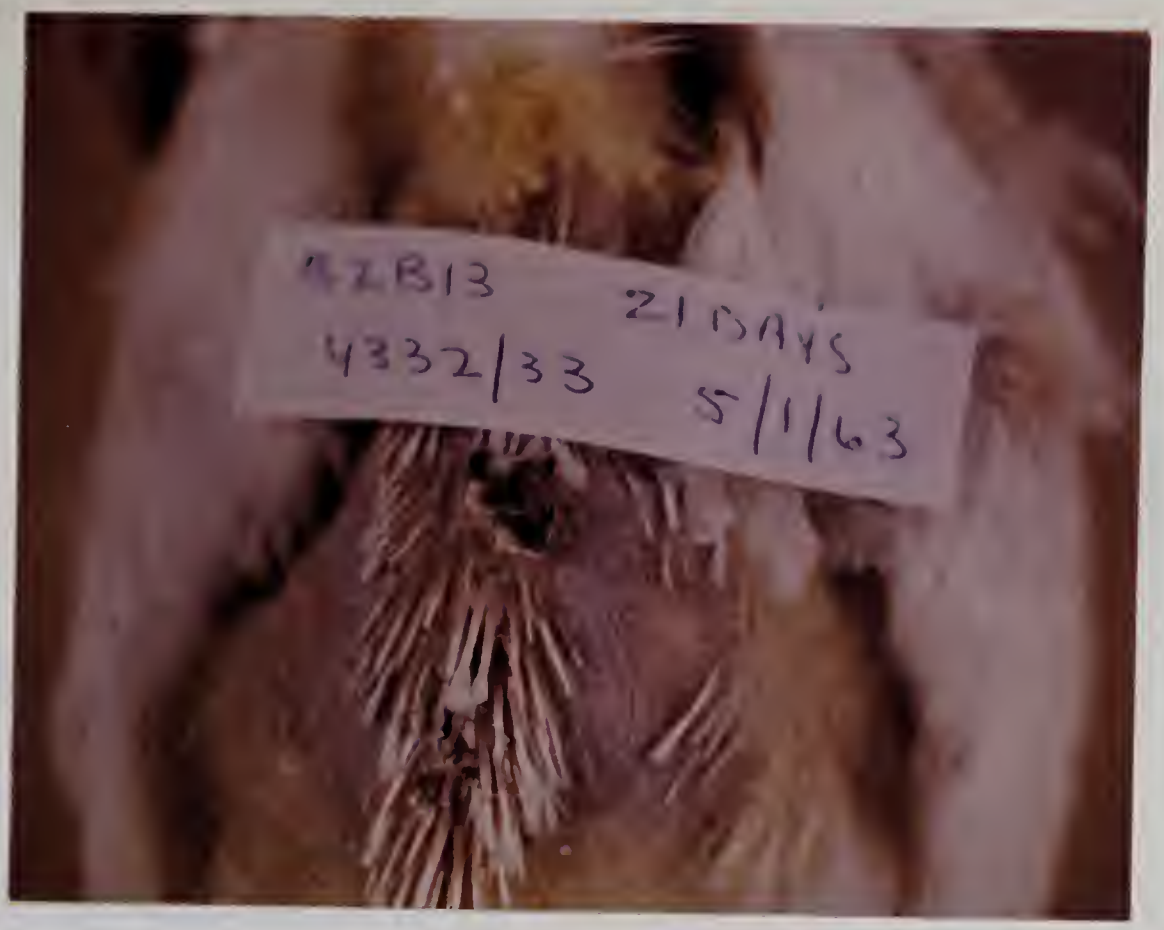


Photograph #5



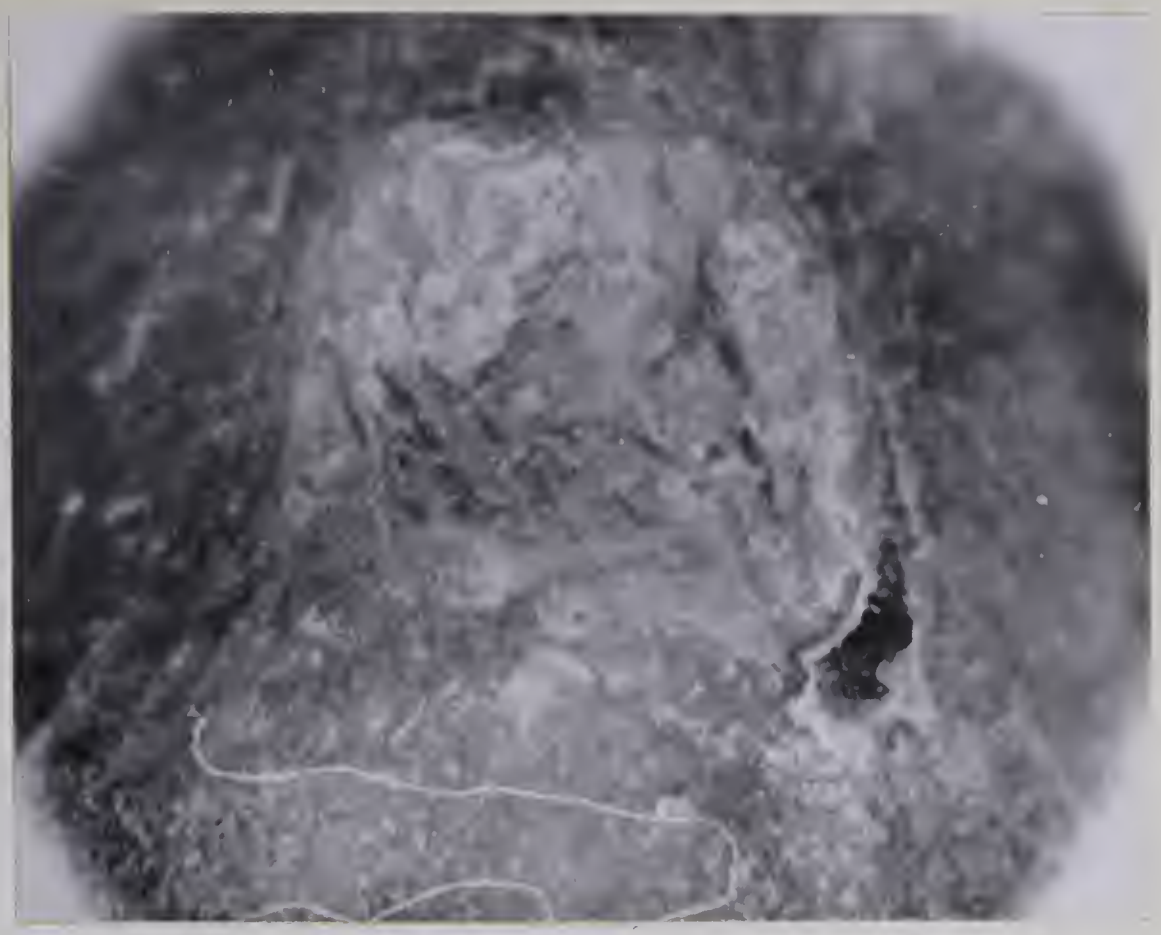
Photograph #6

SKIN GRAFTS

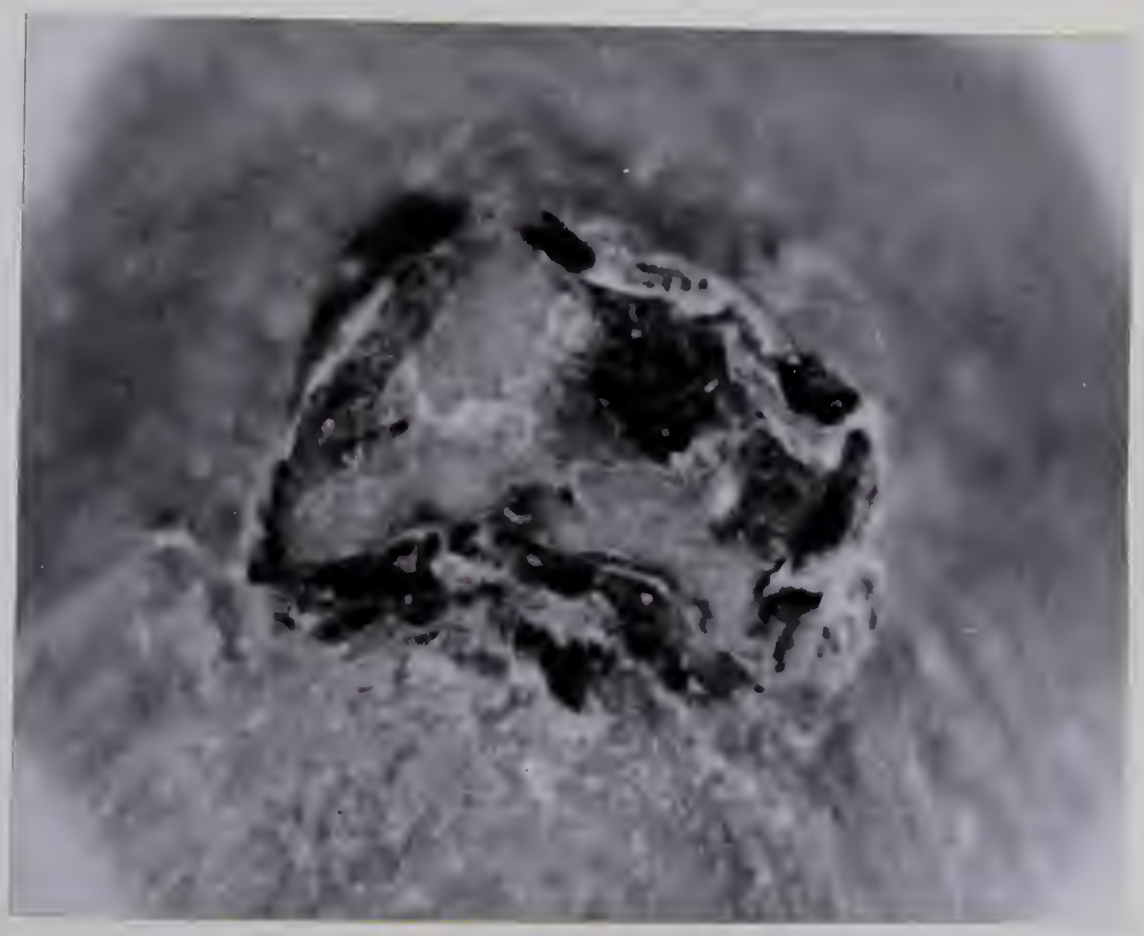


Photograph #7

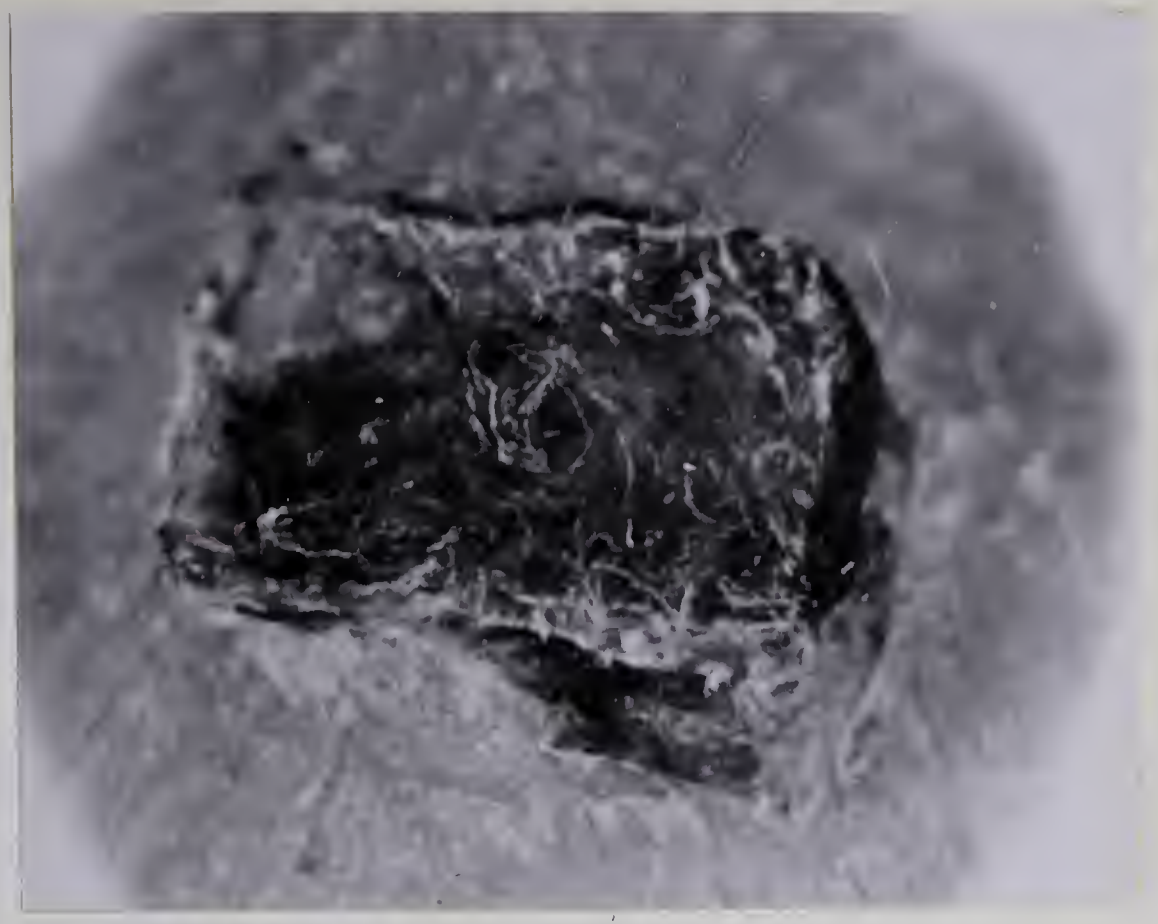
Photograph #8



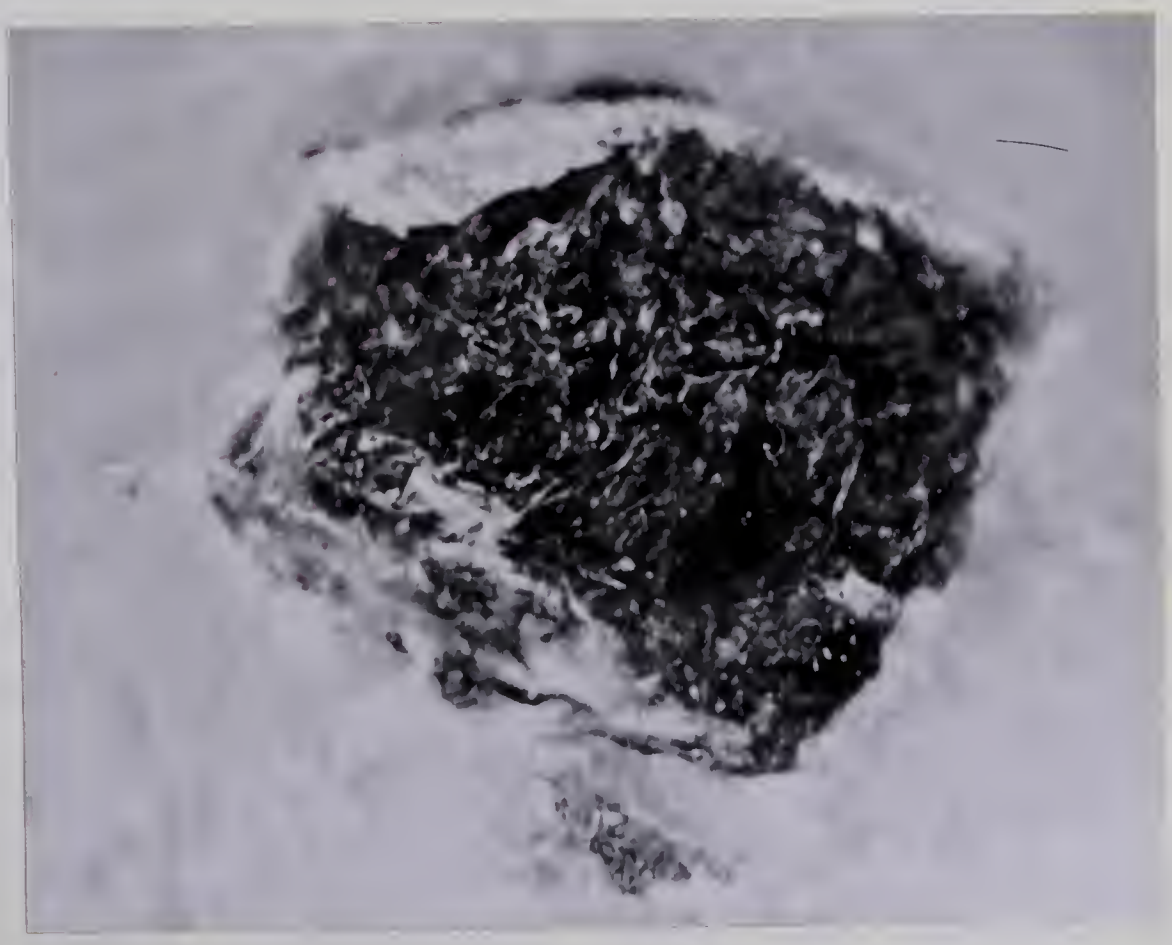
Photograph #9



Photograph #10



Photograph #11



Photograph #12

II. Isoimmunization and Adsorption

Twelve mature, genotyped chickens, selected and described in Table 2 were isoimmunized. Each bird was a recipient of cells from one other bird and a donor of cells to one other bird, except for 7608/7672-182 and 7682/7683-297 which were donors only and 6700/6701-A72 and 7622/7623-A53 which were recipients only. The initial injection was 2 ml. of a 50% suspension of cells. Six days later the isoimmunizations were repeated with 1 ml. injections of the same cell suspensions. After a further six days, each chickens' serum was tested against five types of cells. Washed cells were agglutinated in porcelain agglutination trays and the strength of the reactions graded according to the amount of cell clumping. No attempt at titration of serum was made at this point. The grading system used in Table 2 was as follows:

very weak	weak	strong	very strong
+	++	+++	++++

The unexpected reaction of 6702/6703-210 with $B^{13}B^{13}$ cells and not B^2B^2 cells remains unexplained.

On the basis of these results four chickens with strong reactions, regardless of cross reactivity, were selected for further isoimmunization and their isoantisera titrated using the microagglutination apparatus. Isoantisera were tested on the 12th and 14th days after the initial isoimmunization (Figure 5).

Four pools of adsorbents were prepared by mixing 10 ml. samples of blood and washing the cells. The blood donors were:

Pool No.	1	2	3	4
Donor Genotype	B_1B_1	B_2B_2	$B_{13}B_{13}$	$B_{14}B_{14}$
Donor Identity	6700/6701-A72	7622/6723-A53	6742/6743-41	7747/7748-294
Donor Identity	6659/6660-222	7675/7677-326	6219/6220-168	7608/7672-182
Donor Identity		7686/7687-343	6316/6317-A45	7772/7773-45

Samples of serum (5 ml.) from the 14 day bleedings were adsorbed by adding 10 ml. of packed cells to each sample:

Serum	7772/7773-45	6700/7601-A72	6219/6220-168	7622/7623-A53
	anti B_1	anti B_2	anti B_{14}	anti B_{13}
Pools added	2, 3, 4	1, 3, 4	1, 2, 3	1, 2, 4

The cells and serum were agitated gently on the clinical rotator for 2 hours at room temperature and the agglutinated cells removed by centrifugation. The adsorbed sera were again tested for titre and cross-reactions. Titres are expressed as the reciprocal of the total dilution of the antiserum. The adsorption removed all cross reactivity except in the case of anti B_{14} serum which still cross reacted weakly with B^1 cells. Other titres were reduced by approximately one-half (Figure 5, 14 days).

The chickens were re-immunized with 1 ml. injections of 50% cells in Alsever's solution on the 21st and 25th days after the initial injection and their sera, from the 34th day, retested.

Since the test materials to be used for the immunofluorescent test were from B^2B^2, $B^{14}B^{14}$ and B^2B^{14} chickens, adsorption was carried out on the anti B_{14} and anti B_2 sera only. Two pools of adsorbent cells were prepared.

Pool No.	5		
Donor Genotypes	$B^{13}B^{13}$	B^1B^{14}	$B^{14}B^{14}$
Donor Identity	6742/6743-41 6219/6220-168 6316/6317-A45	6702/6703-210 7490/7491-329 6659/6660-222	7747/7748-294 7608/7672-182

Pool No.	6	
Donor Genotypes	$B^{13}B^{13}$	B^2B^2
Donor Identity	6742/6743.41 6219/6220-168 6316/6317-A45	6722/6723-A53 7686/7687-343 7675/7677-326 7682/7683-297

Packed cells (5 ml.) were added to 13 ml. of isoantiserum for the first adsorption. It was necessary to repeat this procedure four times to remove the cross reactivity. In the fourth adsorption the amount of packed cells was increased to 12.5 ml. A total of 27.5 ml./sample or 2.1 ml. of cells/ml. of antiserum were used. The serum samples recovered after adsorption were slightly diluted. Serum protein concentration dropped from 4.5 to 4.1 gm. % for the anti B_2 serum and from 4.5 to 4.0 gm. % for the anti B_{14} serum as determined by refractometer readings. Cross reactivity was completely eliminated from the anti B_2 serum but as previously the anti B_{14} serum could not be completely adsorbed for B_1 reactivity (Figure 5, 34 days). This serum did not cross react with B_2, the only cross reactivity which could invalidate our results.

The photographs of microagglutination plates show the results of repeating the tests described in Figure 6, (34 days). The column showing the reaction with donor cells, from the bird used to immunize the isoantibody producer, has been omitted in the photographed plates. Instead, the reaction with cells from B heterozygous birds was tested. The letters A, B, C and D represent B^1B^{13}, B^1B^{14}, B^2B^{13} B^2B^{14} cell suspensions used in the columns of wells beneath the letters. The numbers 1, 2, 13 and 14 represent B^1B^1, B^2B^2, $B^{13}B^{13}$ and $B^{14}B^{14}$ cell suspensions used in those columns.

Since there was no more than one unit of titration difference between homozygous and heterozygous cells, having one B isoantigen in common, it was concluded that B isoantigenic strength, in microagglutination tests, was not a direct function of the heterozygous or homozygous condition.

In an effort to assign the effect of adsorption to an electrophoretic region of the serum protein, a semi-quantitative analysis of the sera was done before and after adsorption. Figures 7, 8, 9 and 10 show the microzone electrophoresis adsorption curves. Direct qualitative comparisons of the percentage values may be

made since they are independent of concentration. Quantitative comparisons cannot be made.

In each case the albumin (zones 5-7) has been decreased by adsorption. The main peak in the globulin region (zones 12-14) has become narrower. This may be due to an artifact in the technique or a change in the electrophoretic properties of the proteins in this region, due to adsorption or storage. The most interesting change is the reduction of the small peak (zones 14-15). This peak lies in the gamma globulin region.

Immune electrophoresis analysis was also performed on the same samples before and after adsorption. (Photographs 17, 18, 19 and 20). Adsorption did not consistently remove any of the lines of precipitate evident in this system, which employed goat anti-chicken serum.

B isoantisera, may then, be adsorbed so that it agglutinates erythrocytes of a single B genotype, without major changes in the serum protein constituents, as evidenced by microzone electrophoresis or immune electrophoresis .

TABLE 2

ISOIMMUNIZATION RESULTS

12 days after first injection

Cell Donor	Sex	Genotype	Recipient	Genotype	Expected antiserum	Serum reactions against test cells B_2B_2	B_1B_1	$B_{14}B_{14}$	$B_{13}B_{13}$	Donor
6702/6703-210	M	B^1B^{14}	7747/7748-294	$B^{14}B^{14}$	Anti B_1	-	++	-	-	Not tested
7490/7491-329	M	B^1B^{14}	7772/7773-45	$B^{14}B^{14}$	B_1	-	++	-	-	+++
7747/7748-294	F	$B^{14}B^{14}$	6316/6317-A45	$B^{13}B^{13}$	B_{14}	-	+	+	-	++
7608/7672-182	M	$B^{14}B^{14}$	6219/6220-168	$B^{13}B^{13}$	B_{14}	-	+	+++	-	+++
7772/7773-45	F	$B^{14}B^{14}$	6742/6743-141	$B^{13}B^{13}$	B_{14}	-	+	+	-	++++
7686/7687-343	M	B^2B^2	6702/6603-210	B^1B^{14}	B_2	-	-	-	++	+++
7675/7677-326	M	B^2B^2	7490/6491-329	B^1B^{14}	B_2	++	-	-	-	++
7682/7683-297	M	B^2B^2	6700/6701-A72	B^1B^{14}	B_2	++++	-	+	-	Not tested
6316/6317-A45	F	$B^{13}B^{13}$	7622/7623-A53	B^2B^2	B_{13}	-	+	+	+++	++++
6219/6220-168	F	$B^{13}B^{13}$	7686/7687-343	B^2B^2	B_{13}	-	-	+++	+	++++
6742/6743-41	M	$B^{13}B^{13}$	7675/7677-326	B^2B^2	B_{13}	-	+	+	+	+

ISOIMMUNIZATION AND ADSORPTION

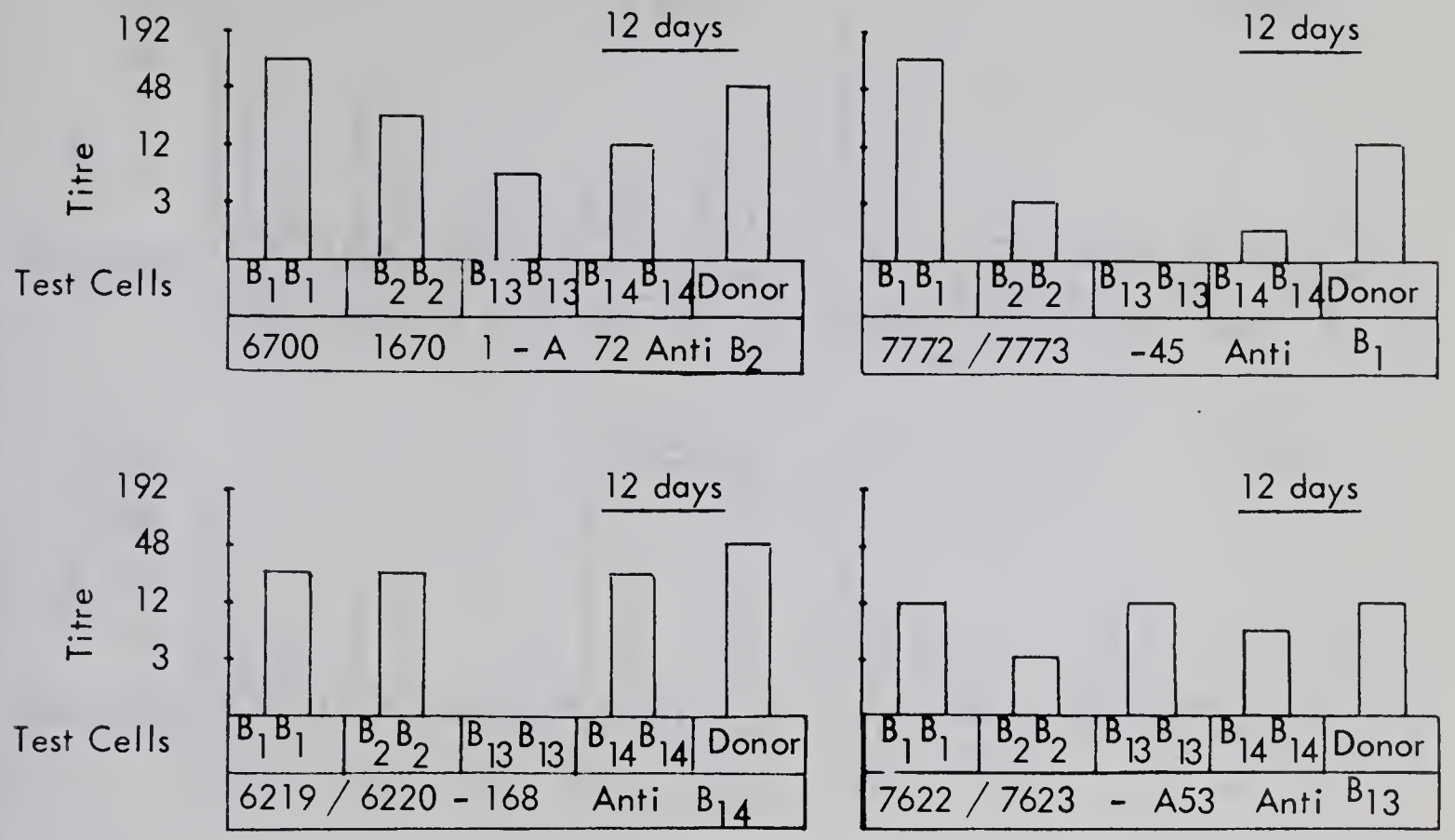
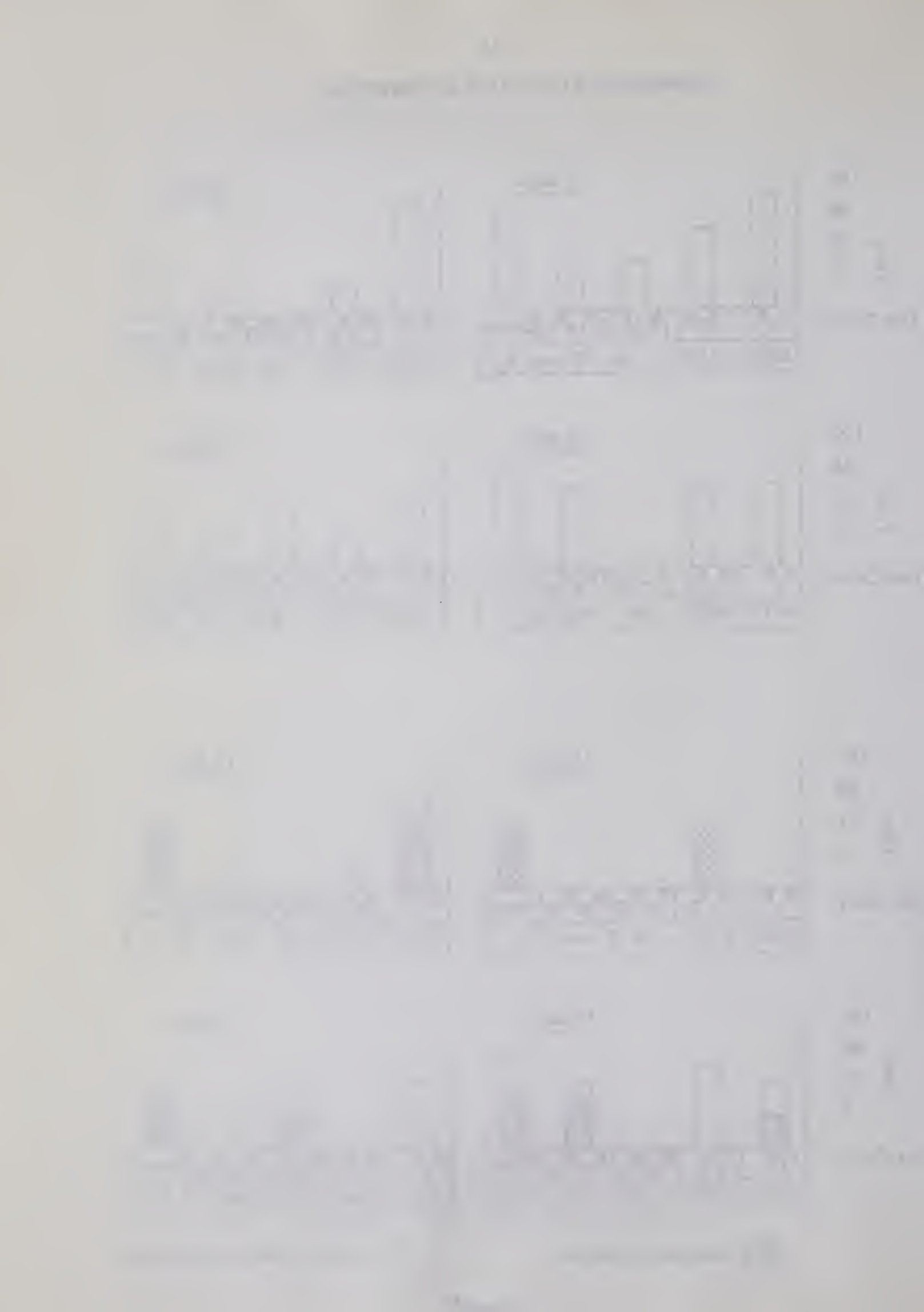
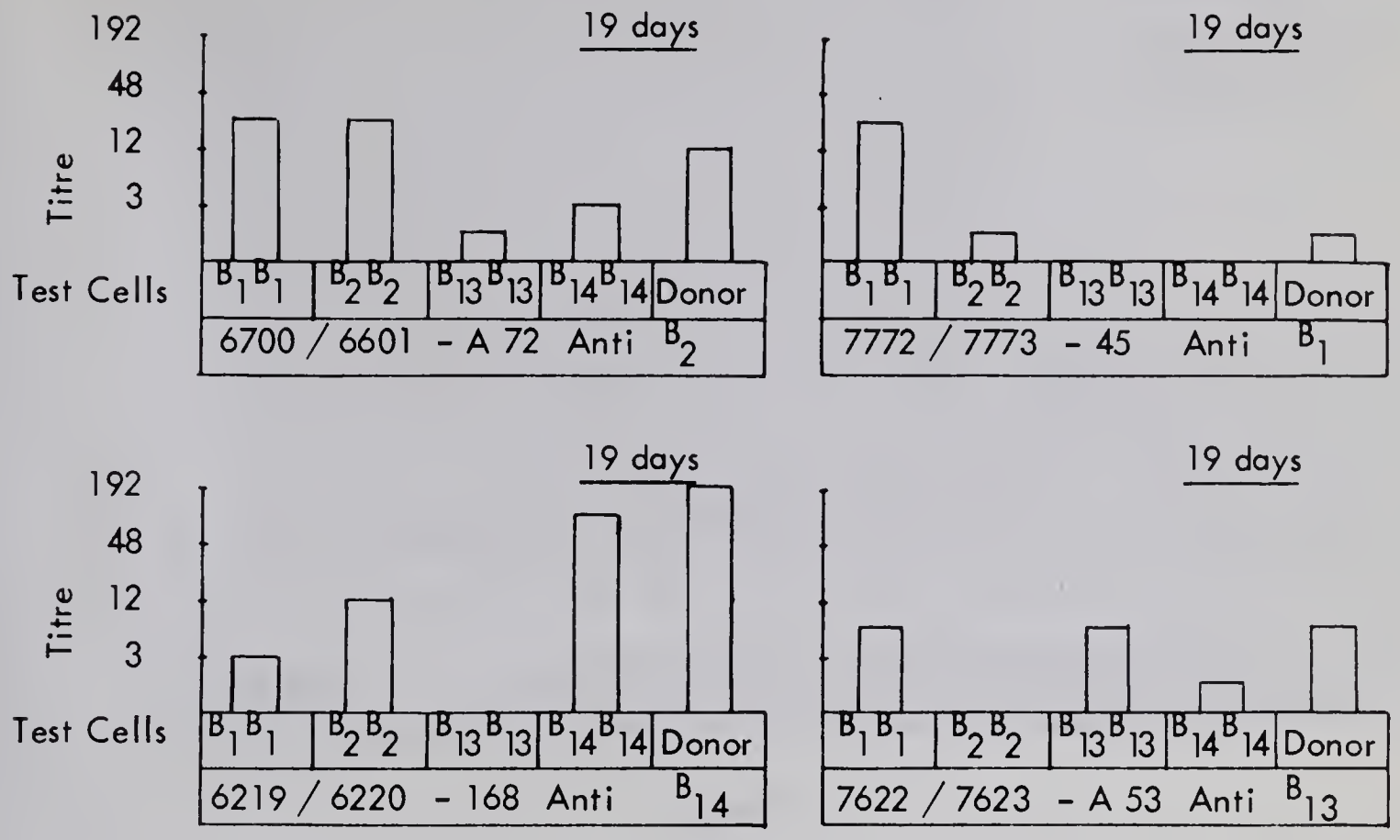


Figure 5



ISOIMMUNIZATION AND ADSORPTION



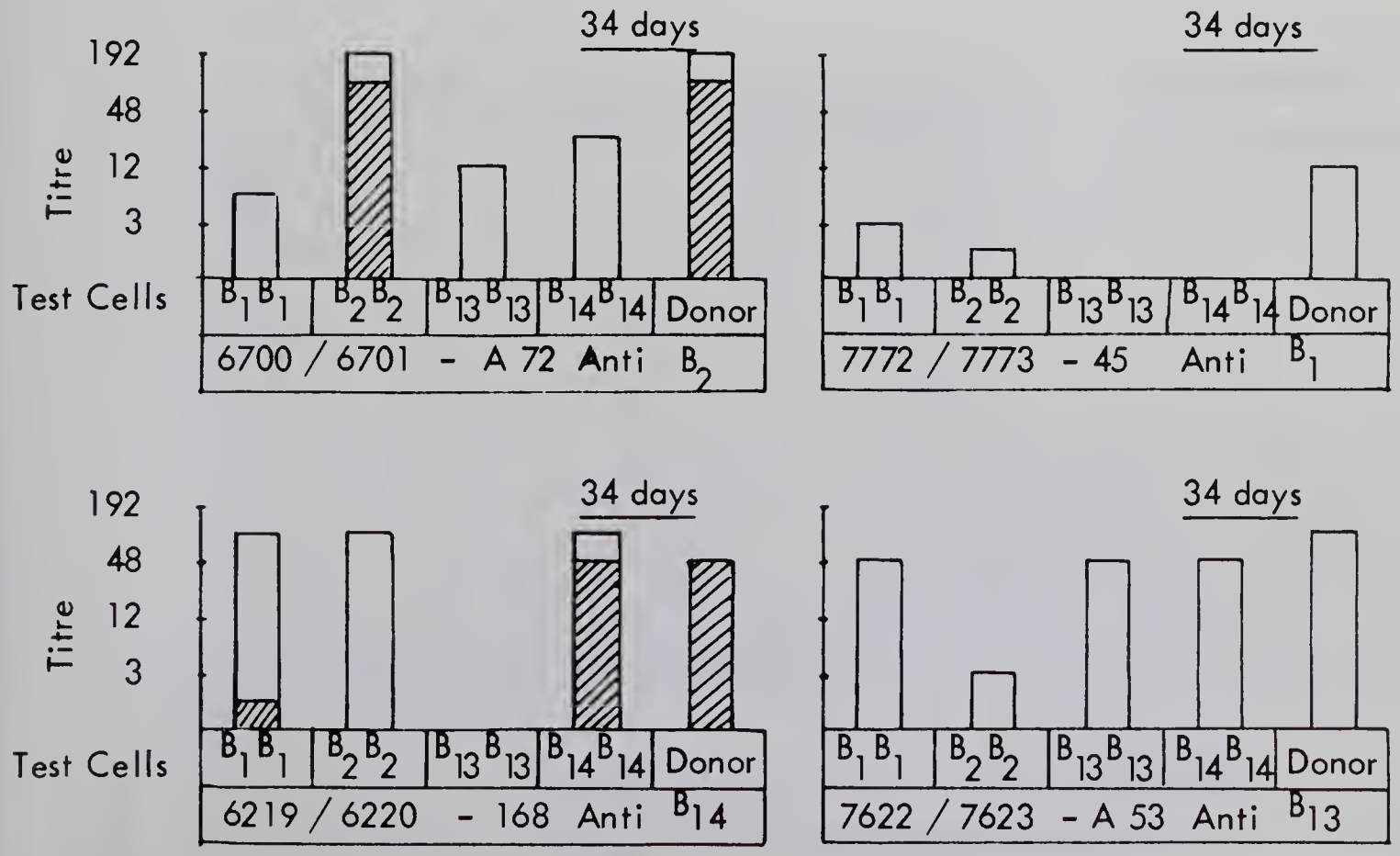


Figure 6

MICROZONE ELECTROPHORESIS ANALYSES OF ADSORBED ANTISERA

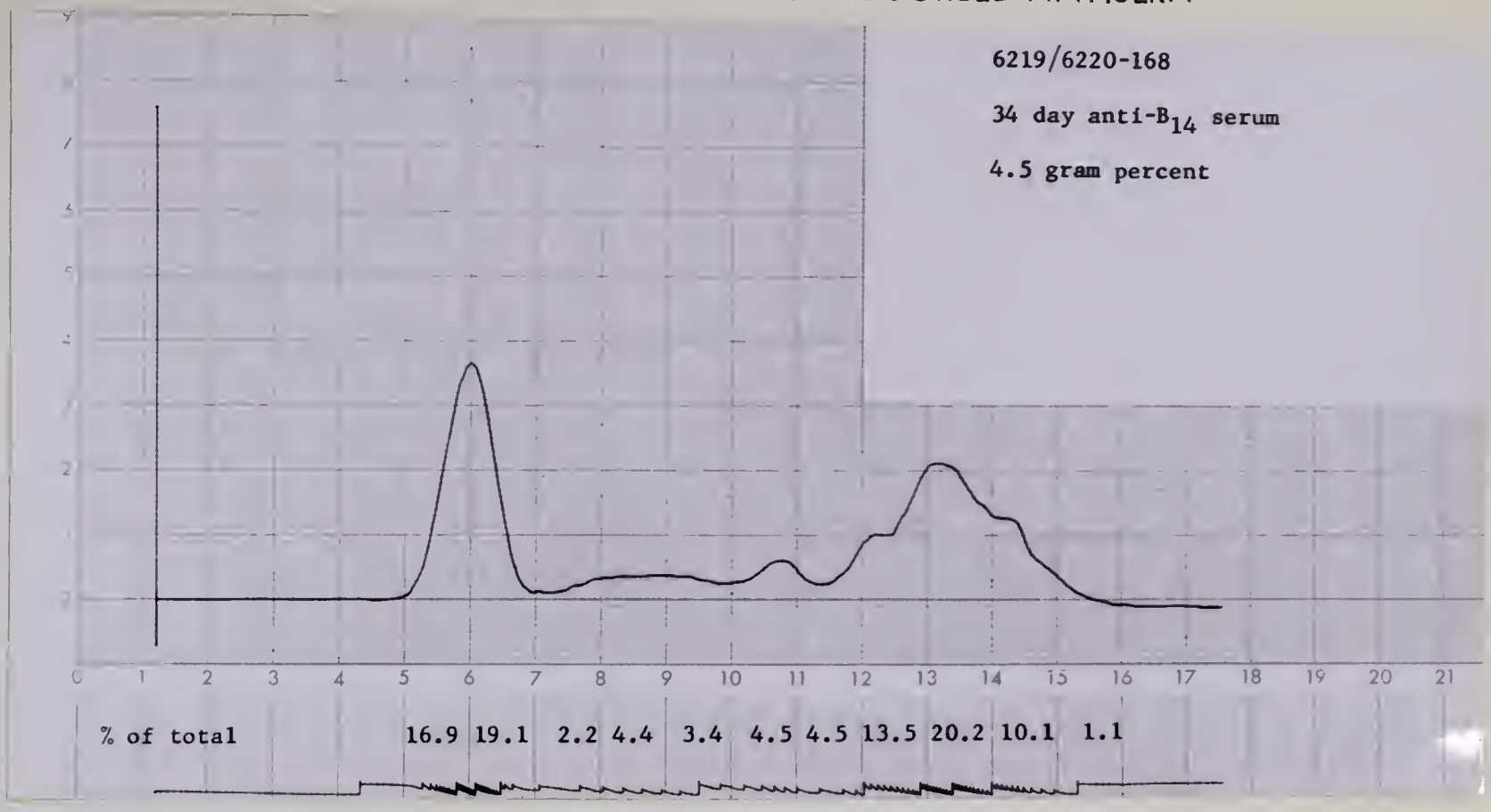


Figure #7

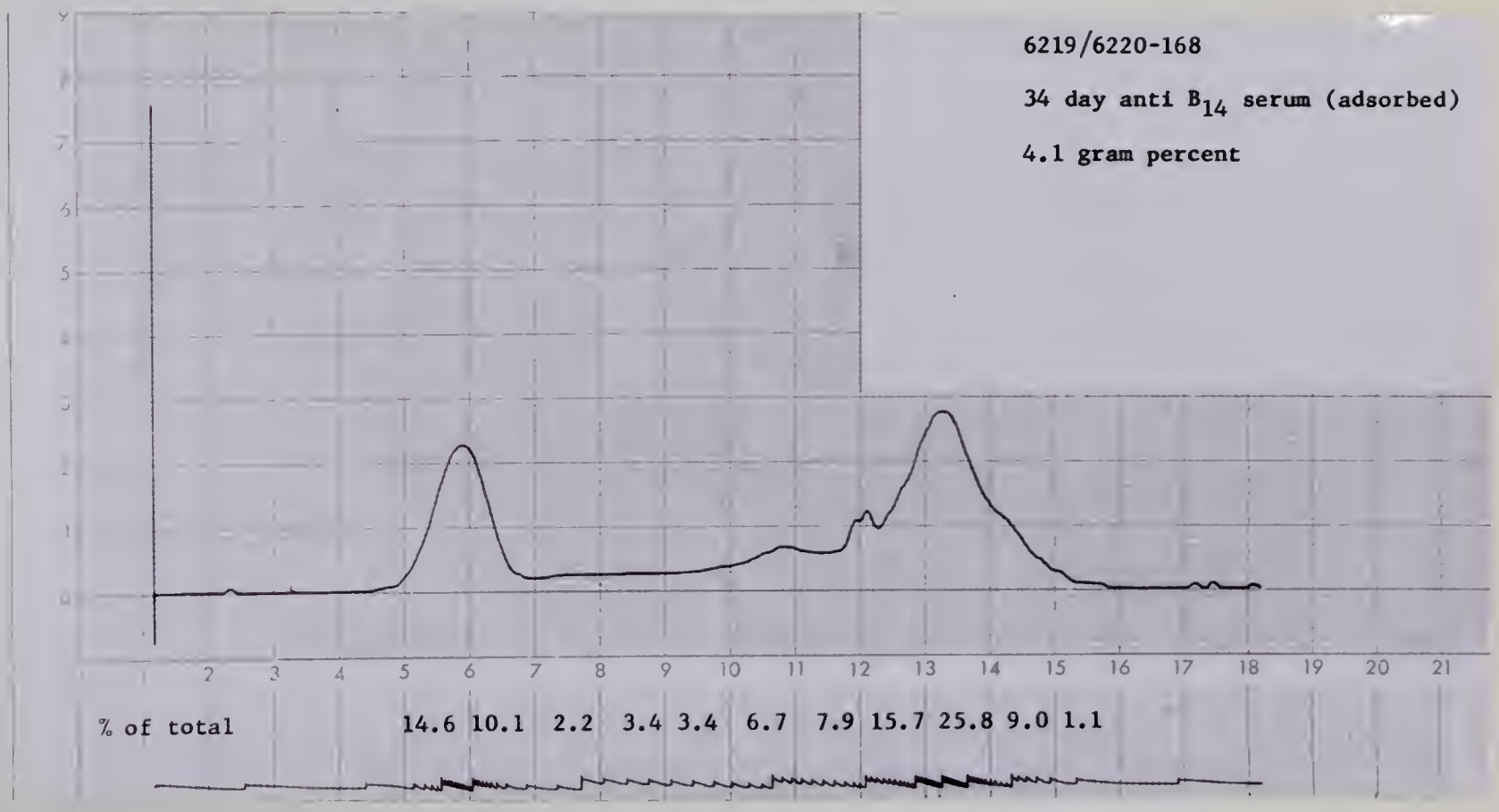


Figure #8

MICROZONE ELECTROPHORESIS ANALYSES OF ADSORBED ANTISERA

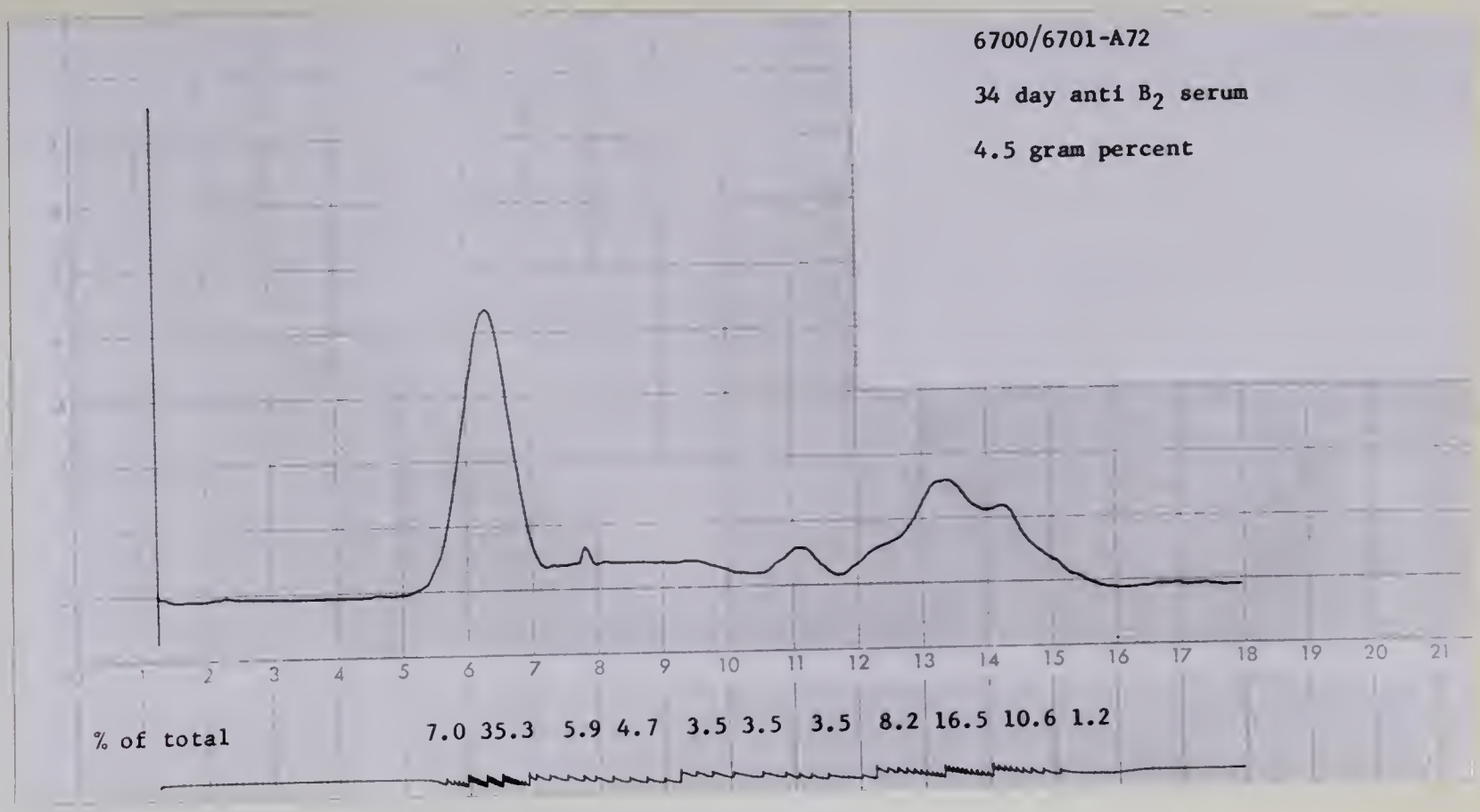


Figure #9

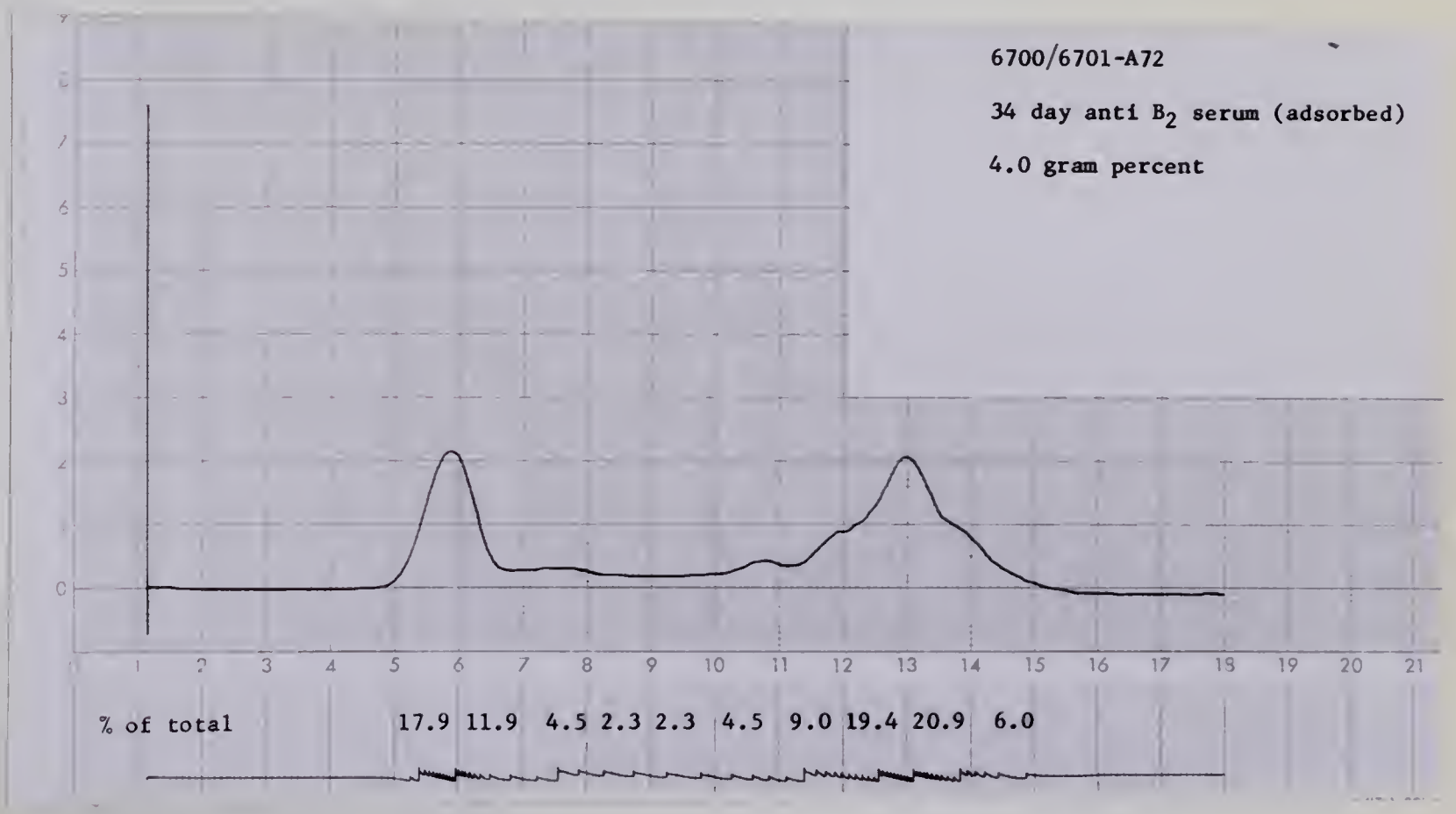
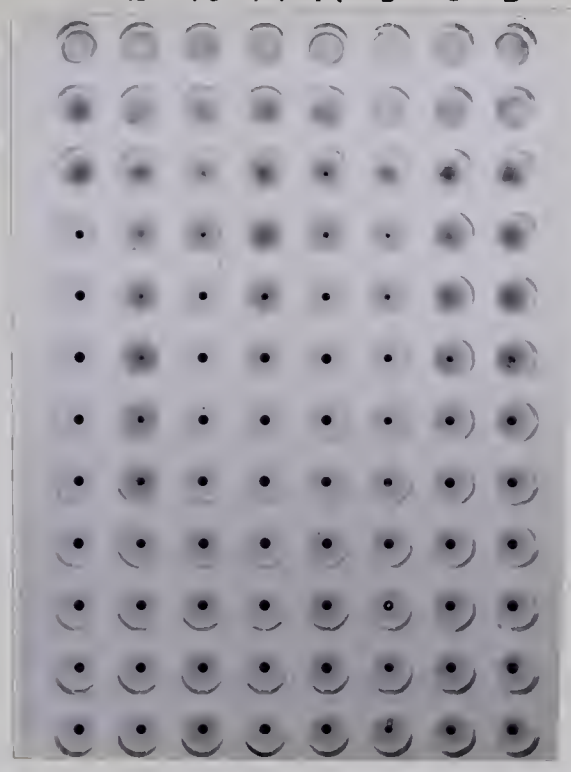


Figure #10

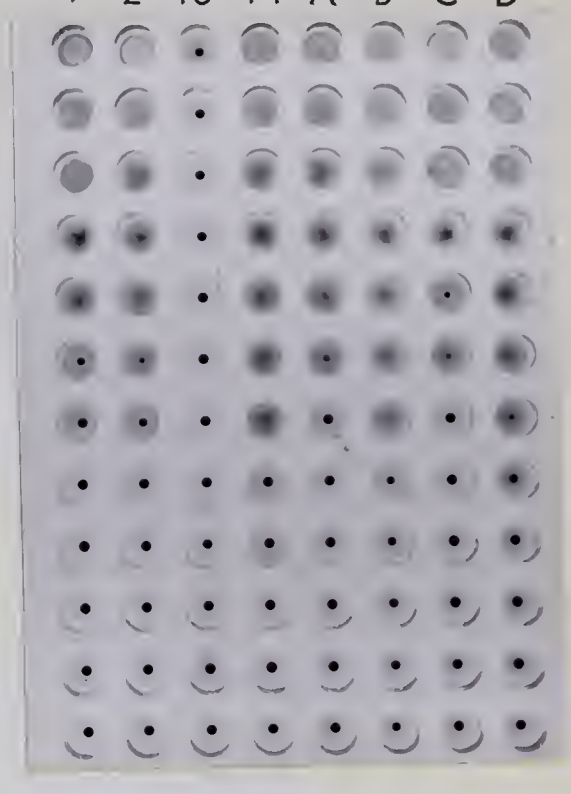
MICROAGGLUTINATION OF ADSORBED ISOANTISERA

6700/6701-A72
Anti B_2 Serum



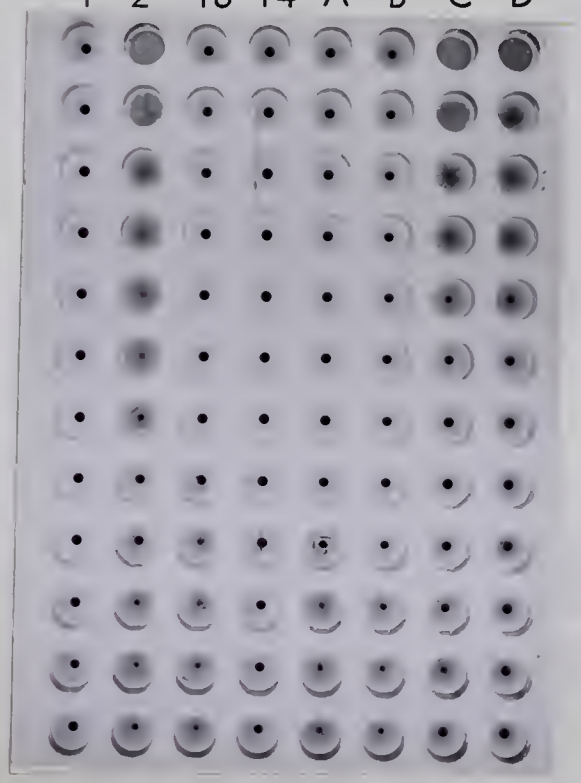
Photograph #13

6219/6220-168
Anti B_{14} Serum



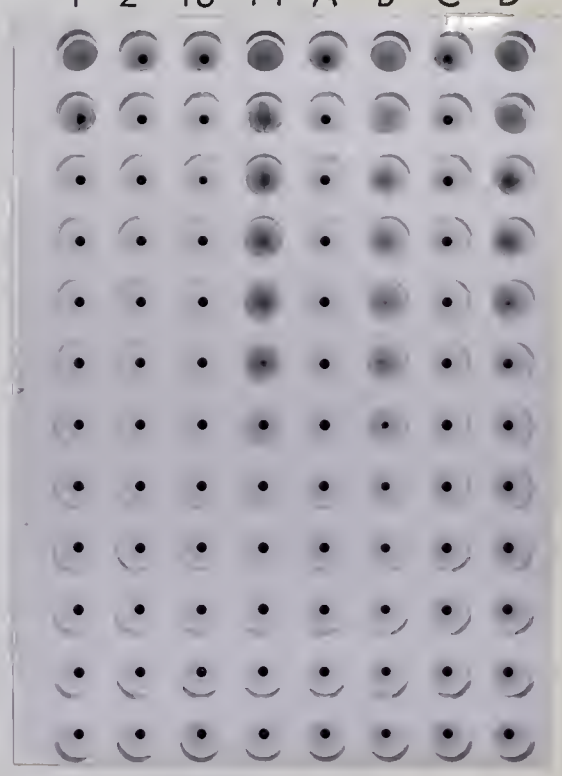
Photograph #14

6700/6701-A72
Adsorbed Anti B_2 Serum



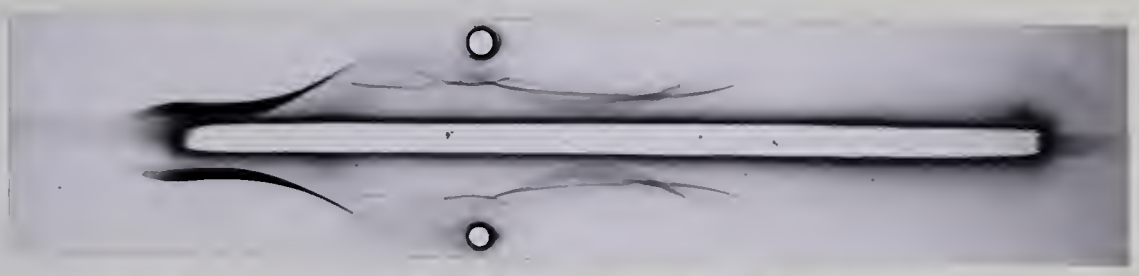
Photograph #15

6719/6220-168
Adsorbed Anti B_{14} Serum

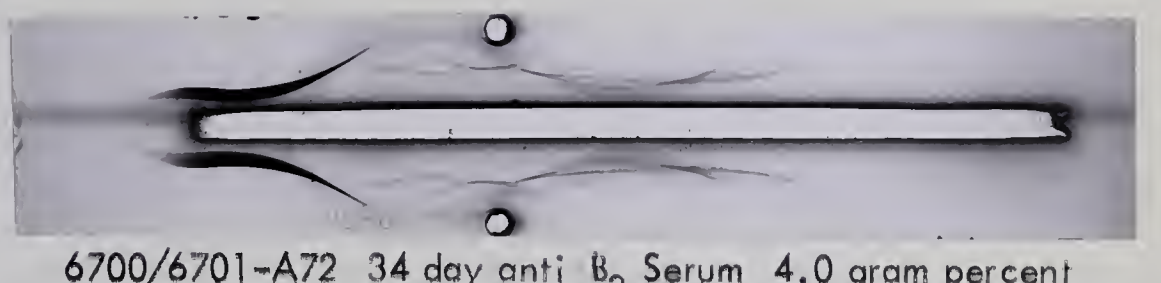


Photograph #16

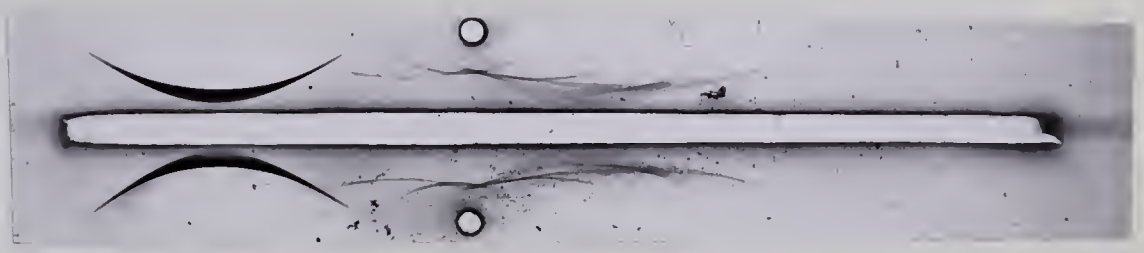
IMMUNE ELECTROPHORESIS ANALYSES OF ADSORBED ANTISERA



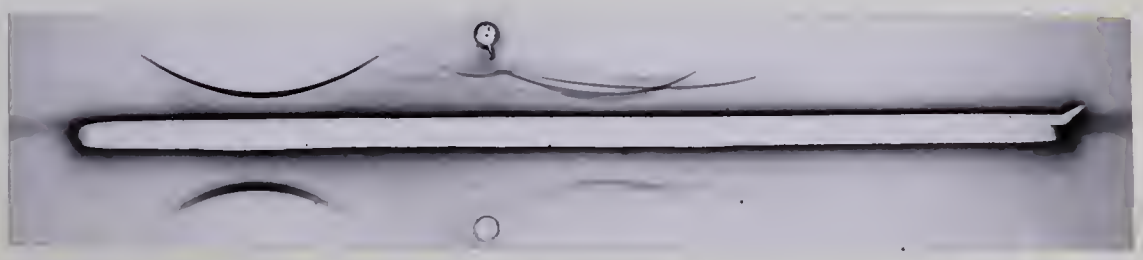
6700/6701-A72 34 day anti B_2 Serum 4.5 gram percent
Photograph #17



6700/6701-A72 34 day anti B_2 Serum 4.0 gram percent
Photograph #18



6219-6220-168 34 day anti B_{14} Serum 4.5 gram percent
Photograph #19



6219-6220-168 34 day anti B_{14} Serum 4.1 gram percent
Photograph #20

III. Isoimmunization and Preadsorption

Although unwanted reactions could be largely removed by adsorption with the appropriate cell types, it is a lengthy procedure. In order to reduce or eliminate completely the production of such unwanted reactions B heterozygous chickens were immunized as follows:

Cell Donor		Foreign Isoantigen	Expected Isoantiserum
4826/6146-81 (B^2B^{14})	6349/6350-34 (B^1B^{14})	B_2	anti- B_2 non-reactive with B_1 and B_{14}
6349/6350-34 (B^1B^{14})	8470/9336-199 (B^1B^{13})	B_{14}	anti- B_{14} non-reactive with B_{13} and B_1

In order to produce as strong a reaction as possible the recipients were given two or three closely spaced injections, allowed to rest for a longer period, then given another series of injections. The closely spaced injections were repeated three times and agglutination tests were made on the 30th and 101st days (Figure 11). Each injection consisted of 1 ml. of a 50% suspension of washed cells in Alsever's solution, given on the following schedule: days 1, 6 and 23, days 54 and 65 and days 87, 99 and 107.

Chicken 6349/6350-34 produced an isoantiserum which did not react with $B^{14}B^{14}$ or $B^{13}B^{13}$ cells in the 30 day test, but reacted with B^2B^2 cells. 8470/9336-199 reacted with all three cell types tested. B^1B^1 cells were not used due to a small number of B^1B^1 chickens. For the 101st day tests the $B^{13}B^{13}$ chicken used previously had died. The B^1B^{13} cells used were from 8470/9336-199 so that the negative reaction shown in part 4 represents isoantiserum from 8470/9336-199 tested against its own cells.

Preadsorption, then, permitted the production of anti-B_2 serum which does not react with B_{14} cells, but the converse is not true. Anti-B_{14} serum, produced in a similar way, cross reacted with B_2 cells. Had it been possible to immunize a B^2B^{13} bird with B^2B^{14} cells this cross reaction might have been avoided. This objective was, however, attained by a more complicated selection of genotypes.

ISOIMMUNIZATION AND PREADSORPTION

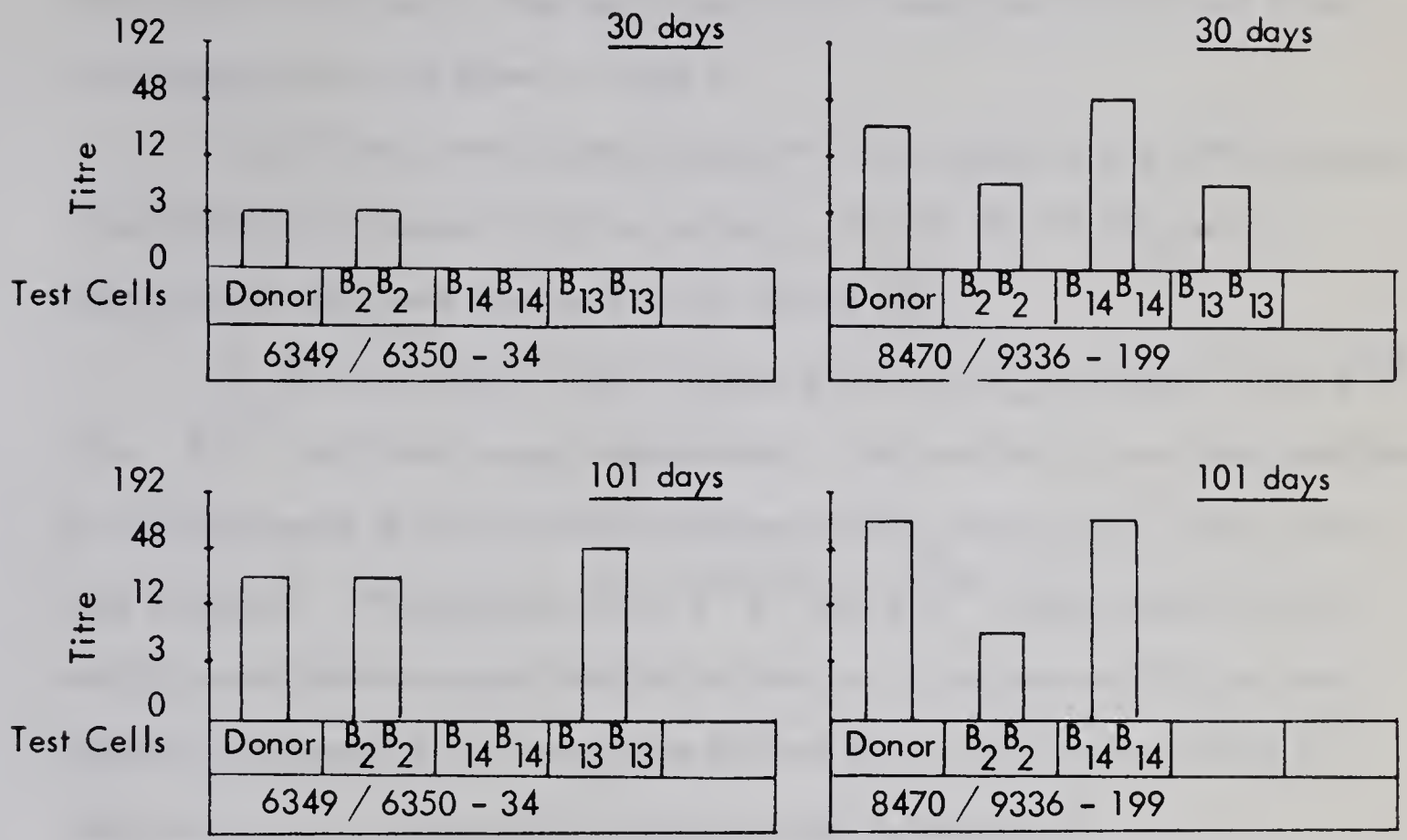


Figure #11

IV. Isoimmunization and Genotypic Matching

Three donor-recipient pairs were selected from the stock of chickens genotyped at seven loci. Their genotypes and the genotypes of cells used to test for isoagglutination are shown in Table 3.

The chickens were isoimmunized with 1 ml. injections of a 50% suspension of washed cells in Alsever's solution on days 1, 12, 21, 32, 43, 55 and 63. Agglutination tests were done on days 23, 40 and 102.

As can be seen from Figure 12, none of the anti B_{14} sera agglutinated B^2B^2 cells. B^1B^{13} cells were strongly agglutinated. This reaction is tentatively ascribed to the high degree of cross reactivity between anti-B_{14} serum and B_1 cells, as was seen previously. The agglutination of $B^{14}B^{14}$ and B^1B^{13} cells in low titre by the anti-B_2 serum remains unexplained but may be due to the presence of Z_2 or other unknown isoantigens on the immunizing and test cells. The reaction with B^1B^{13} cells may be due to cross reactivity between anti B_2 sera and B_1.

The results from the three methods of immunization suggest that the most practical method of producing a concentrated isoantiserum which would agglutinate cells of a single B genotype would be to combine all three methods. That is, to immunize chickens so that isoantigens at loci other than B, which are present in the donor, are also present in the recipient. The recipient should be heterozygous at the B locus so that it is unlikely to produce isoantibodies which cross react with either of the two alleles it possesses. Finally, the resultant isoantisera may be adsorbed free of any unwanted reactivity with washed cells of the appropriate genotype.

TABLE 3

ISOIMMUNIZATION AND GENOTYPIC MATCHING

donor	7608/7682-182	A^2A^6	$B^{14}B^{14}$	C^5C^5	D^1D^2	E^8E^{10}	L^2L^2	Z^1Z^1
recipient	6765/6766-A16	A^2A^6	B^2B^2	C^1C^5	D^1D^2	E^8E^{10}	L^2L^2	Z^1Z^2
possible isoantibodies		none	anti-B_{14}	none	none	none	none	none
donor	7772/7773-45	A^2A^6	$B^{14}B^{14}$	C^1C^5	D^1D^2	E^8E^{10}	L^1L^2	Z^1Z^1
recipient	5939/5940-A9	A^2A^6	B^2B^2	C^1C^5	D^1D^2	E^8E^{10}	L^1L^2	Z^1Z^1
possible isoantibodies		none	anti-B_{14}	none	none	none	none	none
donor	7682/7683-297	A^2A^6	B^2B^2	C^1C^5	$D^?D^2$	E^8E^{10}	L^2L^2	Z^1Z^2
recipient	6659/6660-222	A^2A^2	B^1B^{14}	C^1C^5	D^1D^2	E^8E^{10}	L^1L^2	Z^1Z^1
possible isoantibodies		none	anti-B_2	none	none	none	none	anti-Z_2
Test Cells	6587/6586-128	A^2A^2	B^1B^2	C^1C^5	$D^2D^?$	E^8E^{10}	L^2L^2	Z^1Z^1
	7608/7672-182	A^2A^6	$B^{14}B^{14}$	C^5C^5	D^1D^2	E^8E^{10}	L^2L^2	Z^1Z^1
	-199		B^1B^{13}		other loci unknown			

ISOIMMUNIZATION AND GENOTYPIC MATCHING

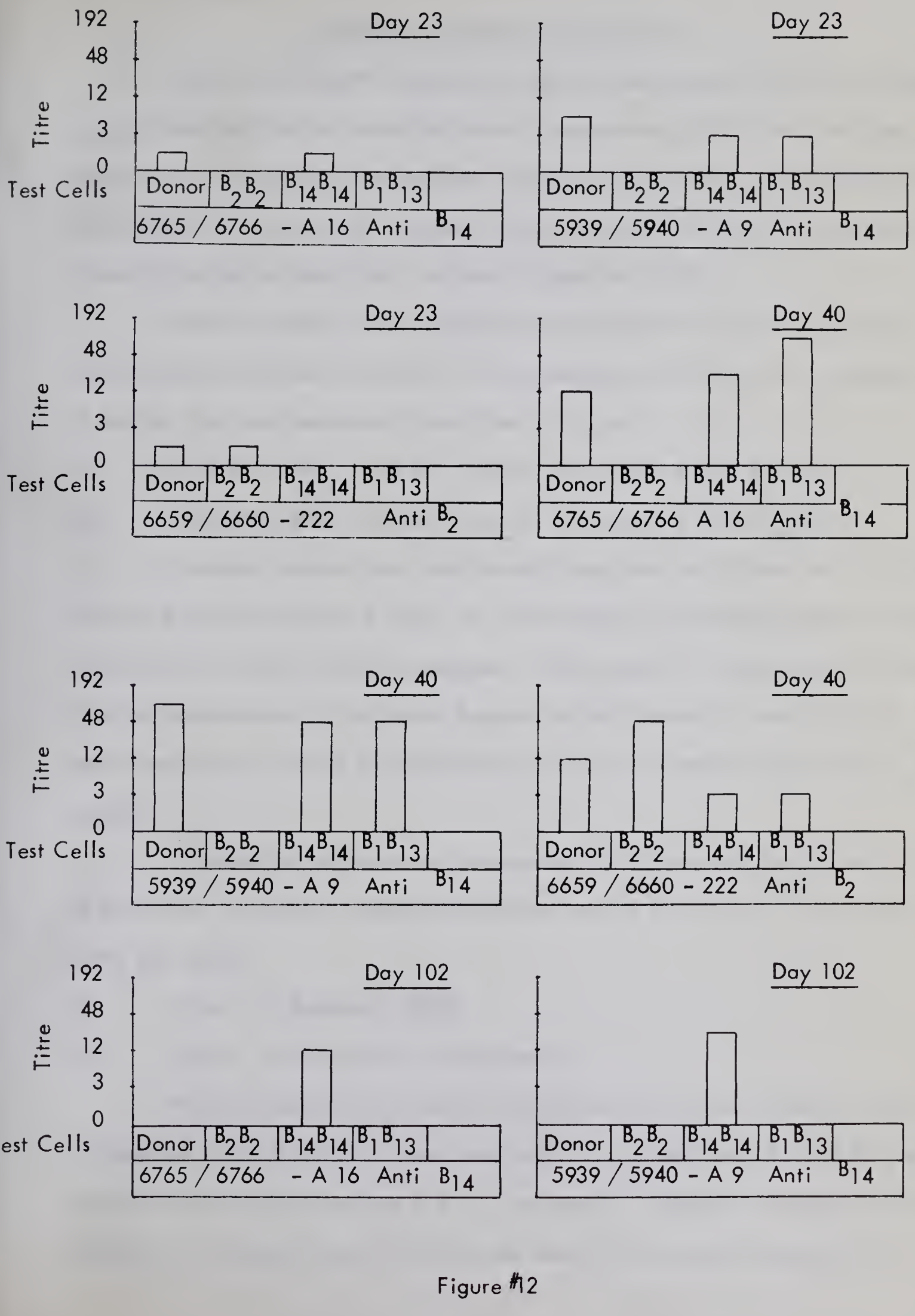


Figure #12

V. Chromatography

1. Ammonium Sulphate Precipitation

Before a fluorescent labelled isoantiserum may be used to trace isoantigens in situ it must be freed of unreacted dye and non-gamma globulin serum proteins which cause non-specific fluorescence. Removal of excess dye, dye impurities and re-buffering of the serum-dye conjugate was achieved rather easily by eluting the crude dye-protein mixture from a column of Sephadex G-25.

The first method used to separate a gamma globulin fraction from serum was ammonium sulphate precipitation. The isoantisera used were 10 ml. samples of the 14 day adsorbed material described in Figure 5.

(1) 6219/6220-168 anti-B_{14}, titre 6, non-reactive with B_2 cells

(2) 6700/6701-A72 anti-B_2, titre 12, non-reactive with B_{14} cells

The serum samples were precipitated three times and diluted with distilled water to a concentration of 4.0 gm. %. The samples were dialyzed against distilled water until the sulphate test was negative. This resulted in a slight amount of precipitate being formed in the dialysis bags which was removed by centrifugation. The serum samples were now at a concentration of 2.0 gm. % and a volume of 4 ml. per sample.

The globulin fractions were conjugated with fluorescent dye in the ratio of 0.05 mgm. dye/mgm. protein and buffered to pH 9.0 with 1 ml. of carbonate buffer per sample.

(1) 4 mgm. of Rhodamine B200

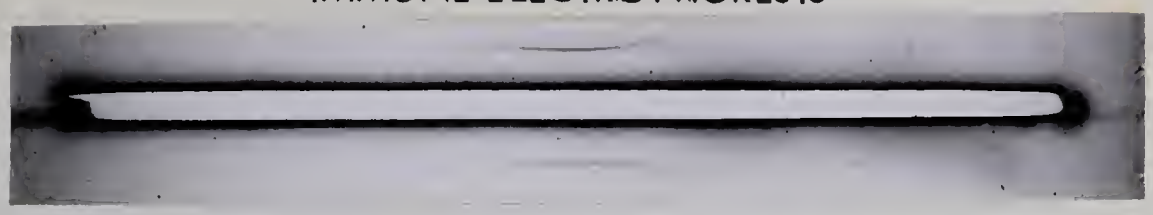
(2) 4 mgm. of Fluorescein Isothiocyanate

After the labelling procedure, the samples were eluted through a column of Sephadex G-25 (11.5 x 2.5 cms.) and buffered at pH 6.0 with 0.0 2M PO_4 buffer. The flow rate of the column was 2.5 ml. per minute. Sample 1 was taken from the refrigerator and pipetted onto the Sephadex bed and the column allowed to run.

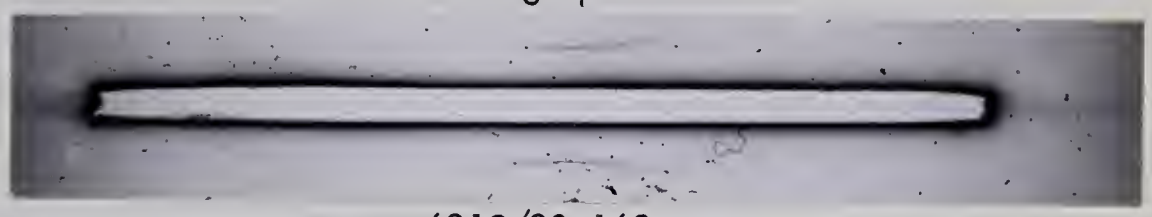
Separation of the reddish-purple, rhodamine labelled globulin from the brown impurities and free dye could be seen clearly with the long-wave ultra-violet lamp. The impurities travelled about 3 inches down the column by the time all the protein had passed through. A total of 24 ml. of fluorescent protein at pH 6.0 was collected in a cylinder in an ice bath after 10 minutes. This dilute protein solution was concentrated in the refrigerated vacuum ultrafiltration apparatus to a volume of 1.8 ml.. (0.75 gm. %) in 10 hours. There was some precipitated protein in the collodion dialysis bag, which was removed. Sample 2 was chromatographed in the same manner but more dilution was encountered. A total of 52 ml. of green fluorescent effluent was collected in 25 minutes. This was also concentrated to 0.75 gm. % in 14 hours. Again precipitates were present which were removed by centrifugation. The concentrated globulin fractions were tested for agglutinin reactivity with fresh cells. They did not agglutinate. That single gamma globulin fractions of the antisera had been separated and labelled with fluorescent dye, is shown by the photographs of the microzone electrophoresis graphs and immune electrophoresis slides which were made immediately following concentration. After storage in the refrigerator for 3 days most of the protein in the solutions had precipitated and the supernatent no longer gave a refractometer reading.

AMMONIUM SULPHATE PRECIPITATION

IMMUNE ELECTROPHORESIS

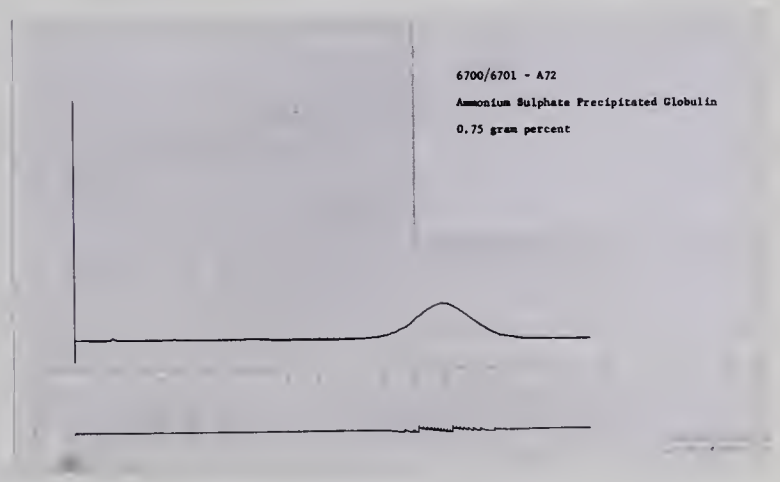


6700/6701-A72
Photograph #21

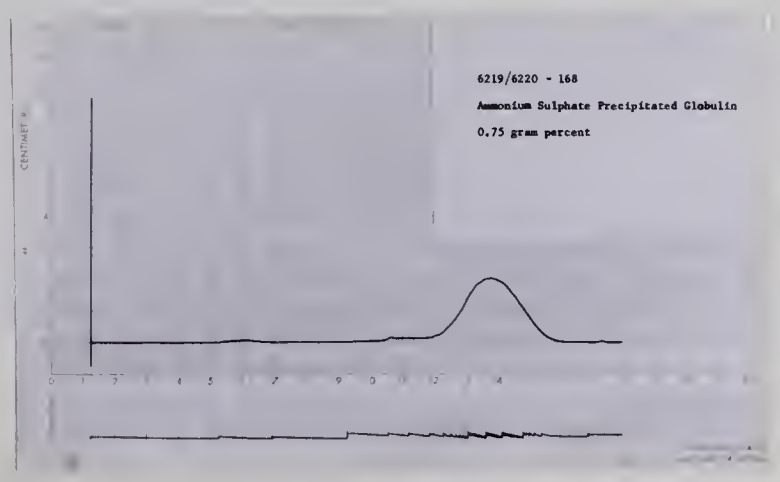


6219/20-168
Photograph #22

MICROZONE ELECTROPHORESIS



Figure#13



Figure#14

2. DEAE

Chromatography on the anion exchanger diethyl aminoethyl ether cellulose (DEAE) was another method used to prepare fluorescent labelled gamma globulins from isoantiserum. Again globulin fractions were tested for purity by immune electrophoresis and microzone electrophoresis and for isoantibody activity by microagglutination.

In the initial chromatographic runs the procedures for fractionation of mammalian sera described by Levy and Sober (1960) and a modification by Curtain (1960) for purification of fluorescein labelled rabbit antiserum were followed. Briefly, the procedure consists of placing a 10 ml. sample of fluorescent dye conjugated serum on top of a 10 x 2.5 cm. column of DEAE. The serum is free of excess dye and buffered with 0.02 M phosphate buffer pH 6.0. The DEAE column is equilibrated with the same buffer. The serum is allowed to sink into the DEAE until it is even with the top of the bed, a buffer reservoir connected to the top of the column and the column allowed to flow. At this pH the DEAE is positively charged. The serum proteins, except for the gamma globulins, are more negatively charged and are adsorbed to the DEAE. The more positively charged gamma-globulins pass through the adsorbent and are collected. The column is then regenerated by removing the DEAE, washing in 0.5 N NaOH to remove proteins, washing in water to restore neutrality, and finally, washing in the buffer solution before repacking.

Five such runs were performed using chicken serum, which does not always respond well to techniques designed for mammalian serum. At pH 6.0 (0.02M PO_4 buffer) the globulin fraction had some affinity for the DEAE and was eluted very slowly, resulting in a thirty to fifty fold dilution. Levy and Sober (1960) report a four to ten fold dilution of mammalian gamma globulins using DEAE buffered at pH 6.0 (0.02M PO_4).

To increase the positive charge of the gamma globulins the pH of the eluting buffer was decreased in stepwise fashion. The run was started with pH 6.0,0.02M PO_4 buffer. After the proteins sank into the DEAE bed and were adsorbed the column was connected to a reservoir of 0.02M PO_4 buffer pH 5.0. The globulin fraction still would not move. When the buffer pH was decreased to 4.6, 0.02M a fluorescent band slowly moved out of the main band and continued to diffuse down the column. This fraction was collected and was approximately a twenty-five fold dilution of the original sample.

The next procedure was to increase the molarity of the eluting buffer while holding the pH at 6.0 in an attempt to replace the weakly bound gamma globulin from the DEAE with phosphate ions. (Peterson and Sober, 1962). This method proved to be the most successful, resulting in a sharp elution of a globulin fraction.

A 10 ml. sample of adsorbed isoantiserum from chicken number 6700/6701-A72, anti B_2, 4.5 gm. %, titre 96, was conjugated with FITC. The sample was freed of excess dye and buffered at pH 6.0, 0.02M by filtering through a 10 x 2.5 cm. column of Sephadex G-25. This resulted in a three fold dilution of the sample to yield 30 ml. of protein solution. It was not necessary to concentrate the sample before DEAE chromatography since at the starting pH and molarity all protein became adsorbed to the DEAE near the top of the column in a fluorescent band about 4 inches wide, no matter what the starting concentration.

A DEAE column was regenerated and repacked to a height of 30 cm. to provide a volume of 120 cc. Buffer (0.02M, pH 6.0) was run through the column until the effluent pH was at 6.0. The sample was introduced and allowed to sink into the DEAE and become adsorbed to it. A reservoir was connected containing 0.1 M PO_4 buffer pH 6.0 and allowed to flow. A fluorescent band immediately became detached and moved down the column. It was collected in six aliquots of 25 ml. each.

The aliquots were concentrated, one at a time, in the ultrafiltration apparatus (Figure 2b).

Aliquot		Time to Concentrate	Refractive Index	Approximate Concentration (gram percent)
1	2 ml.	4 hours 15 minutes	1.3350	0.75
2	2 ml.	2 hours 30 minutes	1.3335	0.50
3	2 ml.	2 hours 45 minutes	1.3334	0.50
4	2 ml.	2 hours 15 minutes	1.3334	0.50
5	1 ml.	3 hours 30 minutes	1.3332	0.50
6	1 ml.	4 hours 30 minutes	1.3334	0.50

Micro Agglutination Test of DEAE Chromatographed Isoantiserum

ALIQUOT NUMBER	1	2	3	4	5	6
	+	+	+	-	-	-
	+	+	-	-	-	-
	+	+	-	-	-	-
	-	-	-	-	-	-
Titre	6	6	2	0	0	0

(Test cells B^2B^2 in all columns)

It should be noted that during concentration slight amounts of precipitate were encountered in each of the aliquots.

The microzone electrophoresis graphs and photographs of the immune electrophoresis slides for each aliquot, which follow, show by two independent methods, that the final fractions contained only gamma globulin and coincide with the microagglutination test for agglutinating isoantibody. The microzone electrophoresis graphs represent a single application of each sample. Goat anti-chicken serum (Lot 1, Colorado Serum Co.) was used for the immune electrophoresis slides. The lines of precipitate were stained for 16 hours. Aliquot 6 has been

omitted as it gave no result with either technique.

Two other such runs were performed, previous to the one reported here, using the same methods. This run was the best that we could achieve using DEAE chromatography. As with the ammonium sulphate method, the labelled globulins tended to form precipitates on storage at 4°C or when frozen at -20°C, and subsequently thawed. This was assumed to be due to the labile nature of chicken globulins when exposed to high salt concentration.

MICROZONE ELECTROPHORESIS
DEAE CHROMATOGRAPHY

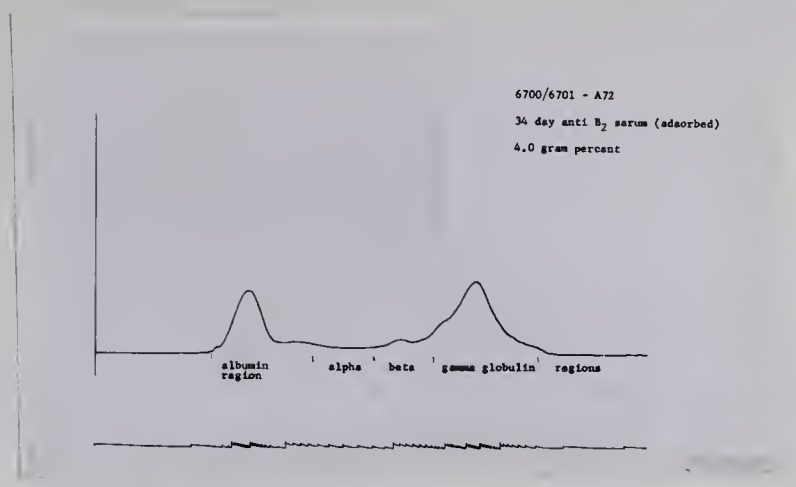


Figure #15

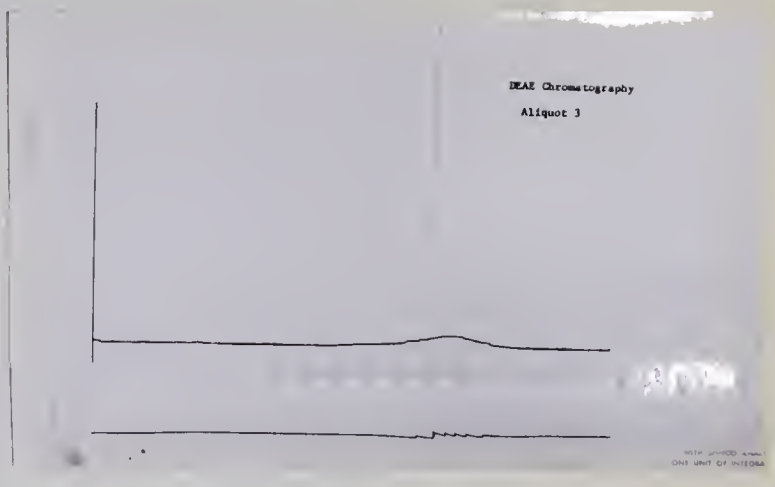


Figure #18

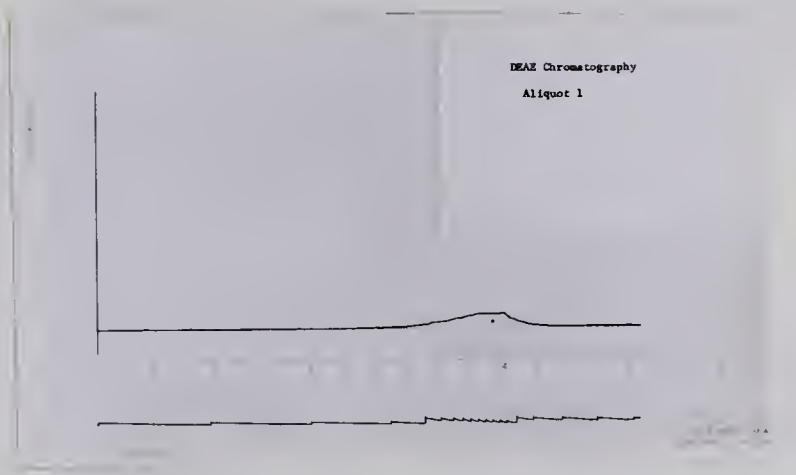


Figure #16

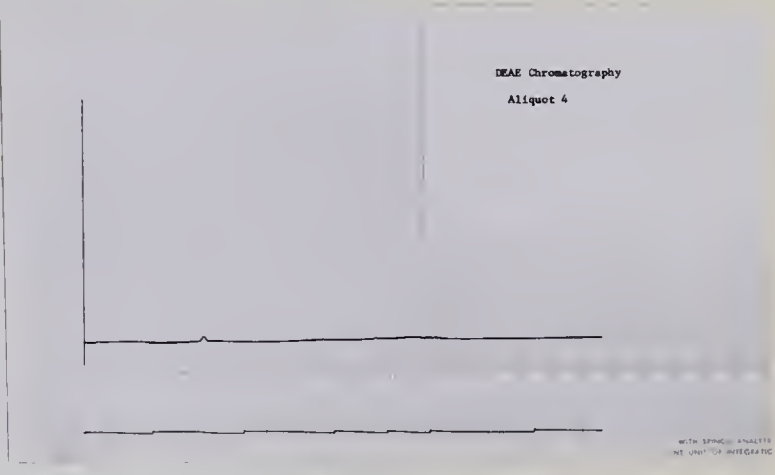


Figure #19

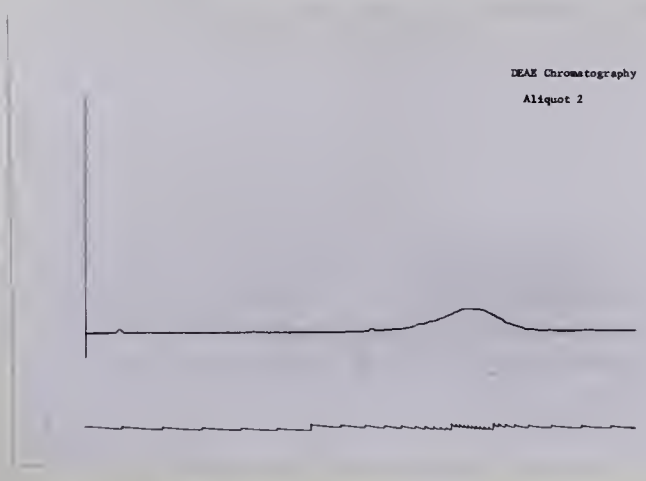


Figure #17

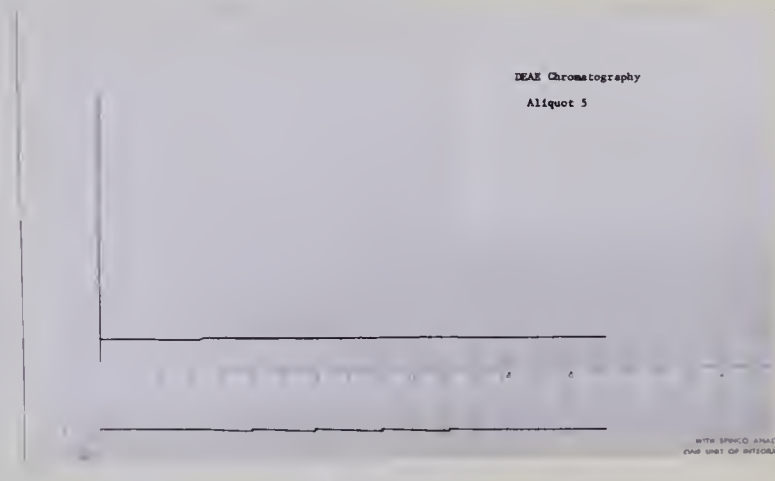
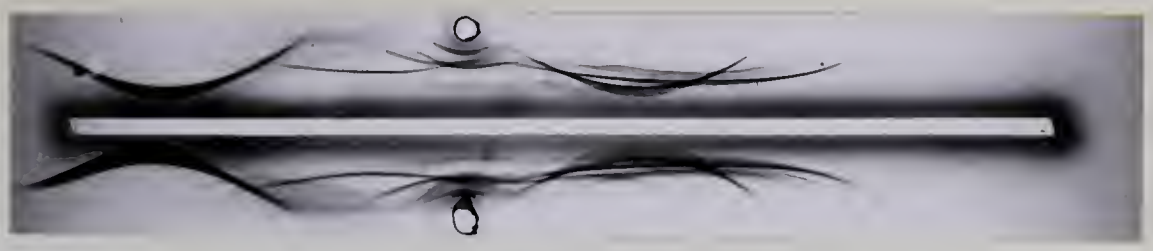
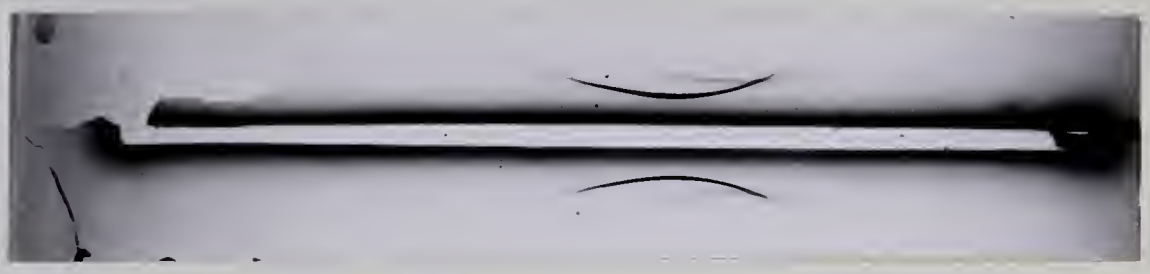


Figure #20

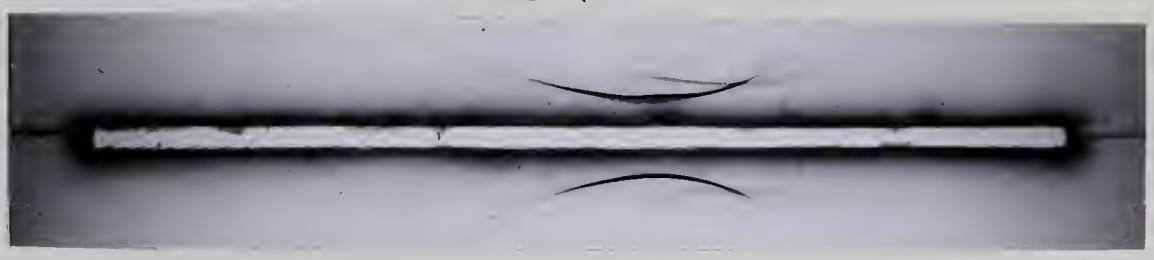
IMMUNE ELECTROPHORESIS
DEAE Chromatography



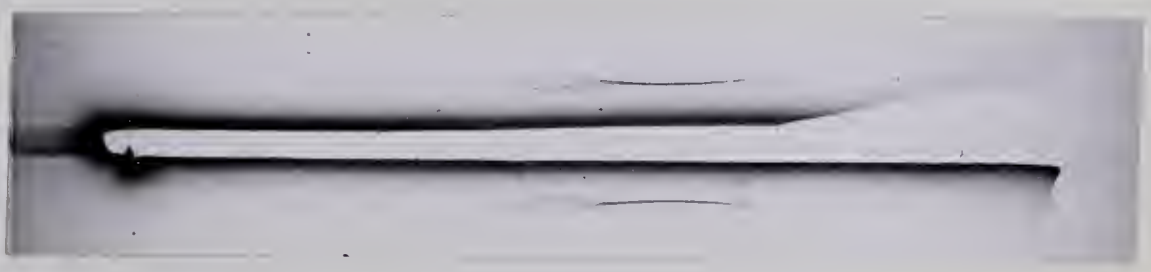
Whole Serum
Photograph #23



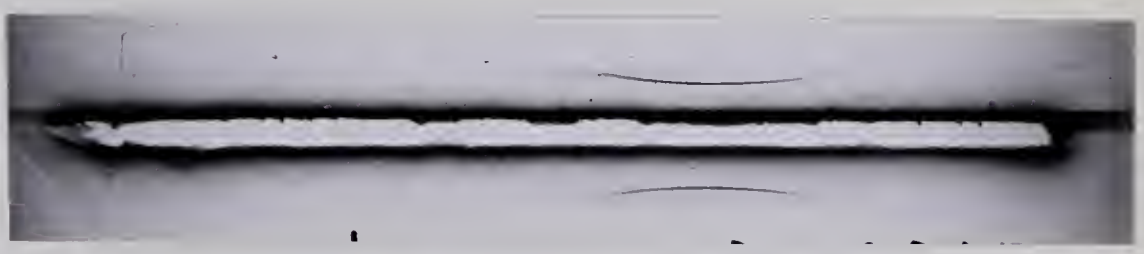
Aliquot 1
Photograph #24



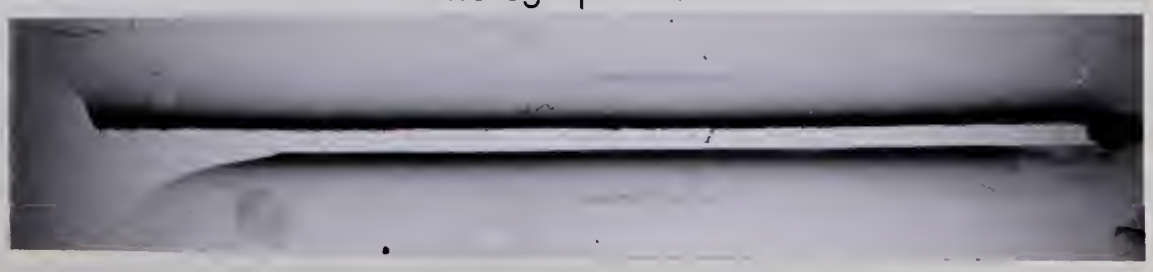
Aliquot 2
Photograph #25



Aliquot 3
Photograph #26



Aliquot 4
Photograph #27



Photograph #28

3. Sephadex G-200

A third method of fractionating serum was Sephadex G-200 chromatography. Sephadex is a hydrophilic dextran which forms a gel of cross linked spherical grains when hydrolyzed and packed in a column. Each grade of Sephadex has a pore size related to the degree of cross linkage within the grains. Sephadex G-25 has a high degree of cross linkage, and large molecules of a molecular weight greater than 25,000, are excluded from entering the gel grains and flow quickly through the column. Smaller molecules, such as salts and dye, enter the grains and move more slowly down the column. Sephadex G-200 operates on somewhat the same principle to sort serum protein molecules into fractions, on the basis of their molecular size. As the protein molecules move down the column, the smallest molecules enter the pores in the gel grains most often and travel the slowest. The larger molecules enter less often and travel faster to be eluted sooner. Molecules of a molecular weight greater than 200,000 are excluded from the gel grains completely and are eluted first. This separates the serum into three main fractions: high molecular weight (19S) globulins, smaller (7S) globulins and non-globulins. (Fahey et al. 1964).

Two columns were packed with Sephadex G-200 to a height of 15 cm. per column and connected in tandem to provide a 100 cc. bed volume. This distributed the weight of the gel onto two surfaces so that the gel would not pack so tightly as to make the flow rate too slow. Sephadex G-25 was packed on top of the G-200 in the upper column to a height of 5 cm. (20 cc. bed volume). The gel was buffered at pH 7.2 with 0.01M PO_4 buffer. The flow rate was 1.2 ml. per minute, 45 ml. of buffer being eluted as the front of the fluorescent protein travelled from the top to the bottom of the column (void volume) in 38 minutes. Ice water was circulated through the cooling jackets of the column for a half hour before starting chromatography. The temperature of the buffer at the outlet was 3°C.

A 1 ml. sample of isoantiserum (6349/6350-34, anti B_2, titre 24, 4.7 gm. %) conjugated with FITC was chromatographed. A strong fluorescent band remained in the upper G-25 portion of the bed throughout the run. The proteins spread out over two-thirds of the length of the column but did not separate into bands. However, slight colour differences could be seen. The lower portion fluoresced a deep apple green while the upper portion was more yellowish.

Starting with the first fluorescent drop the proteins were collected in six, 10 ml. aliquots in graduated tubes in an ice bath. The aliquots were poured into six dialysis reservoirs of the large ultrafiltration apparatus and concentrated for 20 hours, when 1 ml. remained in each bag. The aliquots were removed with a 24 inch long catheter attached to a #20 needle on a 10 ml. syringe. The catheter reached to the bottom of the dialysis bag and all the protein solution could be drawn into the catheter without entering the syringe. The solution was forced gently from the catheter into a labelled serum bottle. Refractometer readings were taken to determine approximate concentration, microagglutination tests were done to determine isoantibody activity, and microzone electrophoresis combined with immune electrophoresis analyses performed to determine the constituent proteins in each aliquot.

Aliquot	Refractive index	Approximate concentration (gram percent)
1	no reading	0.00
2	1.3340	0.50
3	1.3346	0.60
4	1.3352	0.70
5	1.3350	0.70
6	1.3345	0.50

Microagglution Test

ALIQUOT	1	2	3	4	5	6
	+	+	+	-	-	-
	+	+	-	-	-	-
	+	-	-	-	-	-
	-	-	-	-	-	-
Titre	6	3	2	0	0	0

(B^2B^2 cells used in all columns)

Double applications of each sample were used in the microzone electrophoresis analyses. This accounts, at least in part, for the higher peaks.

Immune electrophoresis employed goat anti chicken serum (Lot 2, Colorado Serum Co.) Lines of precipitate were stained for 24 hours. The labelling has been omitted from all immune electrophoresis slides as the coding method used does not coincide with the terminology used in this thesis.

Aliquot 6 is not shown as it contained only a small amount of albumin.

MICROZONE ELECTROPHORESIS
SEPHADEX G-200 CHROMATOGRAPHY

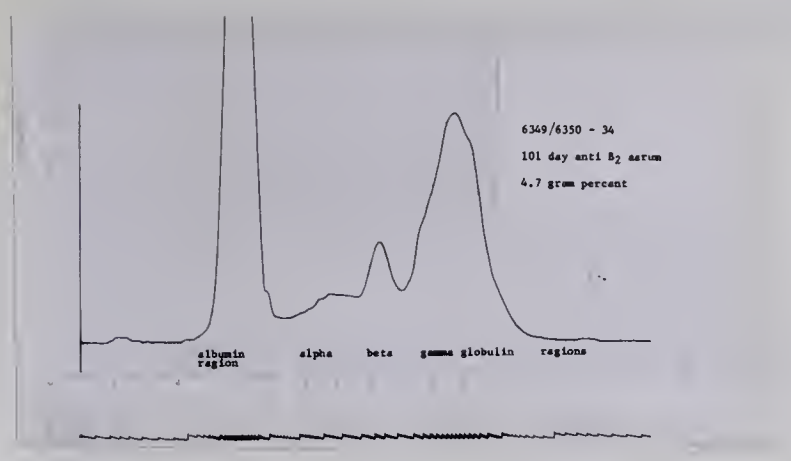


Figure #21

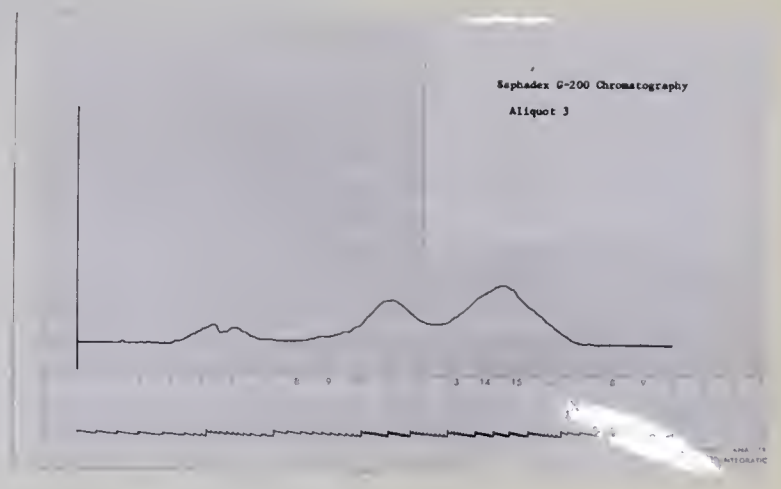


Figure #24

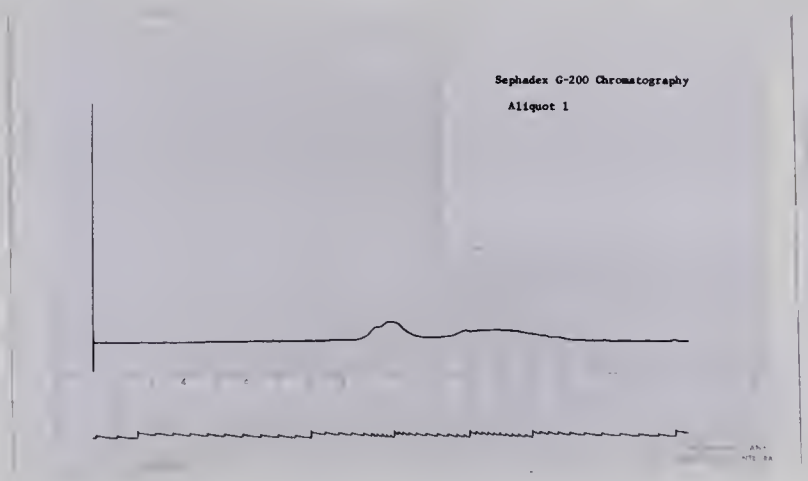


Figure #22

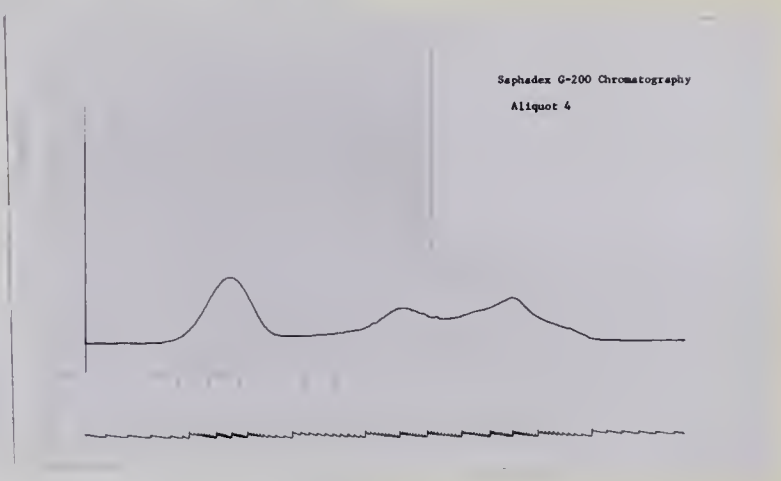


Figure #25

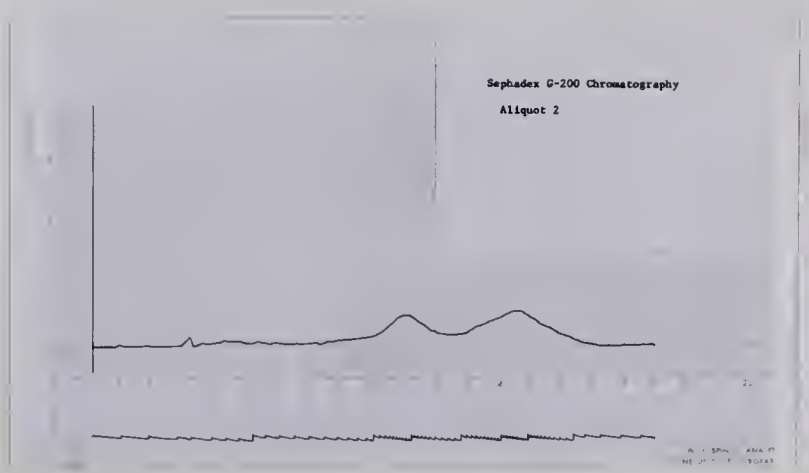


Figure #23

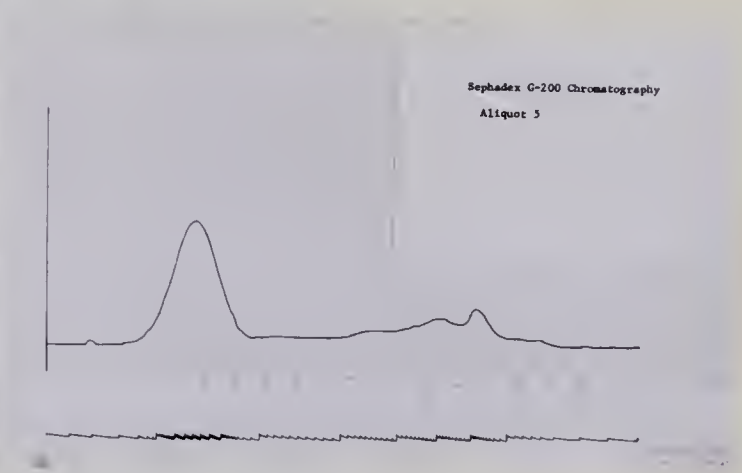
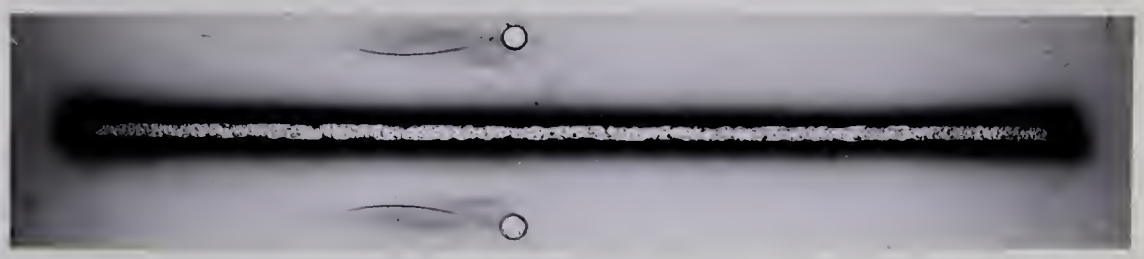


Figure #26

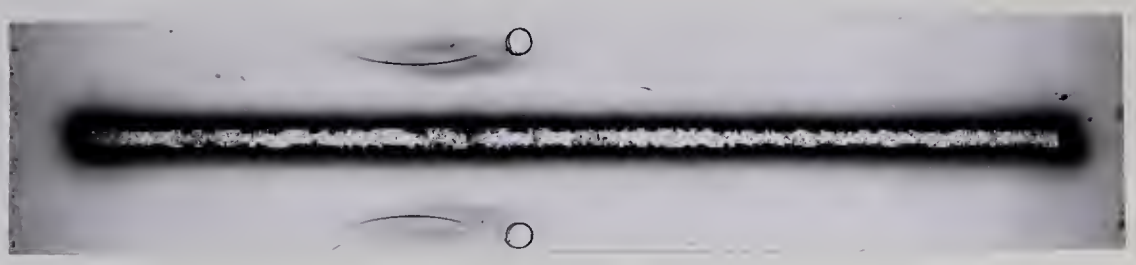
IMMUNE ELECTROPHORESIS

SEPHADEX G-200 CHROMATOGRAPHY

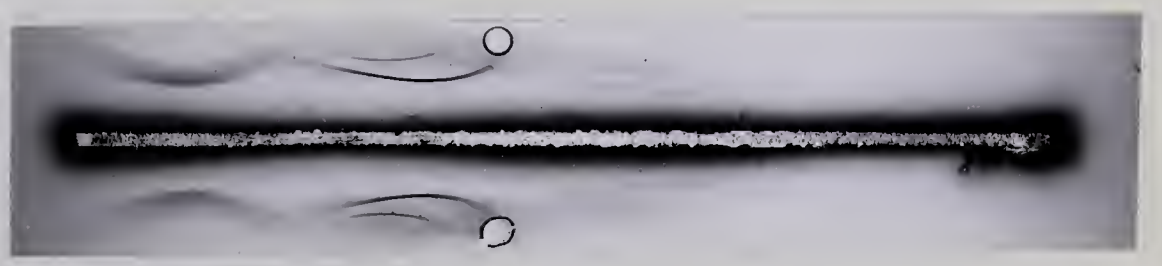
Whole Serum
Photograph #29



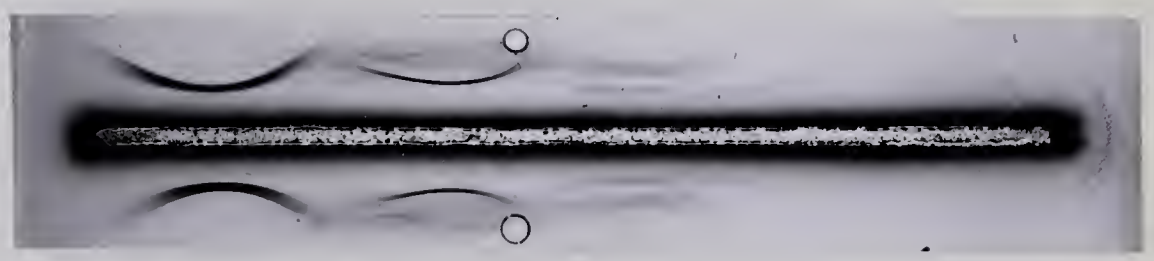
Aliquot 1
Photograph #30



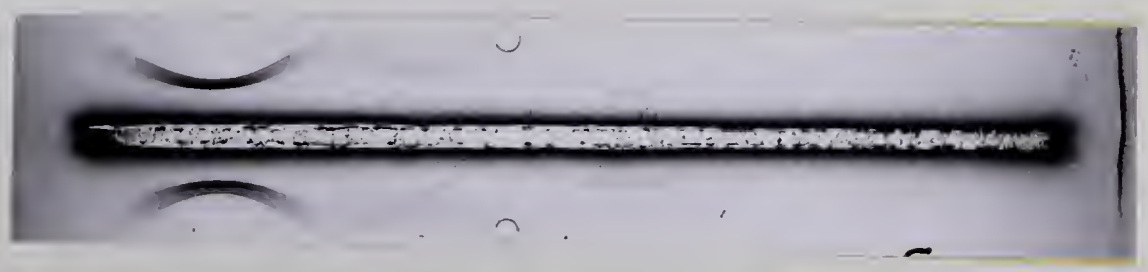
Aliquot 2
Photograph #31



Aliquot 3
Photograph #32



Aliquot 4
Photograph #33



Aliquot 5
Photograph #34

VI. Isoantigen Tracing with Fluorescent Isoantibodies

1. Acridine Orange Staining

Gamma globulin fractions of fluorescent iso-antisera were applied to frozen sections of tissues, blood cell suspensions, and spleen imprints. Photographs 35, 36 and 37 show examples of these three test materials stained with acridine orange. Photographs 38, 39 and 40 are of acridine orange stained cells from a smear of a blood cell suspension, at higher magnification. Such acridine orange stained materials provided a method of viewing test materials under ultra-violet illumination preliminary to labelling the same type of materials with fluorescent antibodies. General morphology, optimum conditions for fluorescent microscopy and the details of colour photomicrography were studied using acridine orange.

In Photograph 35 the outer cutaneous layer of the skin can be seen on the right as a gray-green ribbon. This is underlain by an epithelium of tightly packed cells. The central layer of loosely organized elongate cells have reddish orange cytoplasm (RNA) and yellowish nuclei (DNA). The low resolution of the photograph, as compared to the ones following, reflects the fact that it was taken earlier in the study, using Reichert equipment.

The small green nuclei of erythrocytes without cytoplasmic fluorescence may be seen among the larger spleen cells in Photograph 36. The spleen cells have large yellowish-green nuclei surrounded by orange cytoplasm.

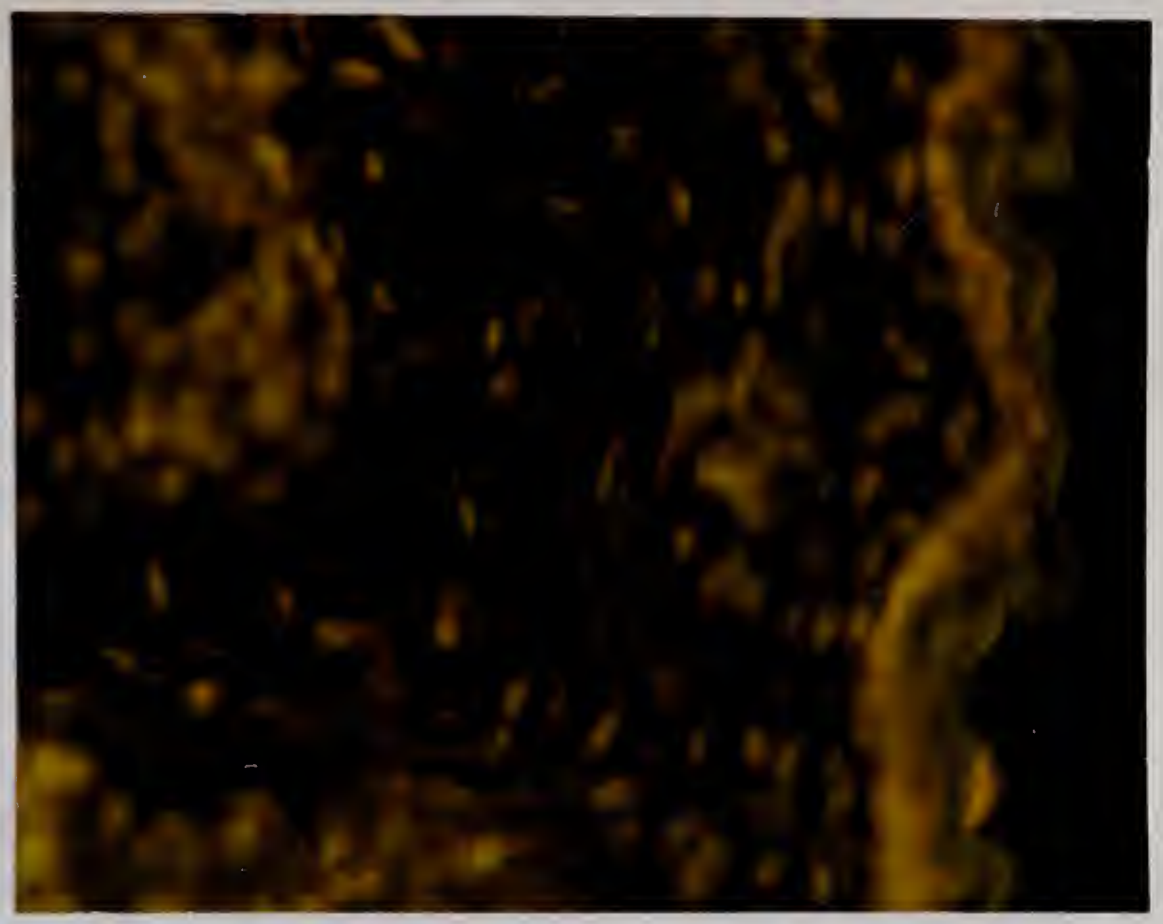
In Photograph 38 erythrocyte nuclei can be recognized by their oval shape. Some large, round, green fluorescing leukocytes without the reddish fluorescence of RNA are present. Others with red fluorescing cytoplasm surrounding the nucleus are lymphocytes.

Organization of nuclear material can be seen in the green fluorescing erythrocyte nuclei in Photographs 38, 39 and 40. The small, round cells with yellow nuclei and red cytoplasm are lymphocytes, other leukocytes are unidentified.

The large cell with a divided nucleus in Photograph 40 is thought to be undergoing mitosis.

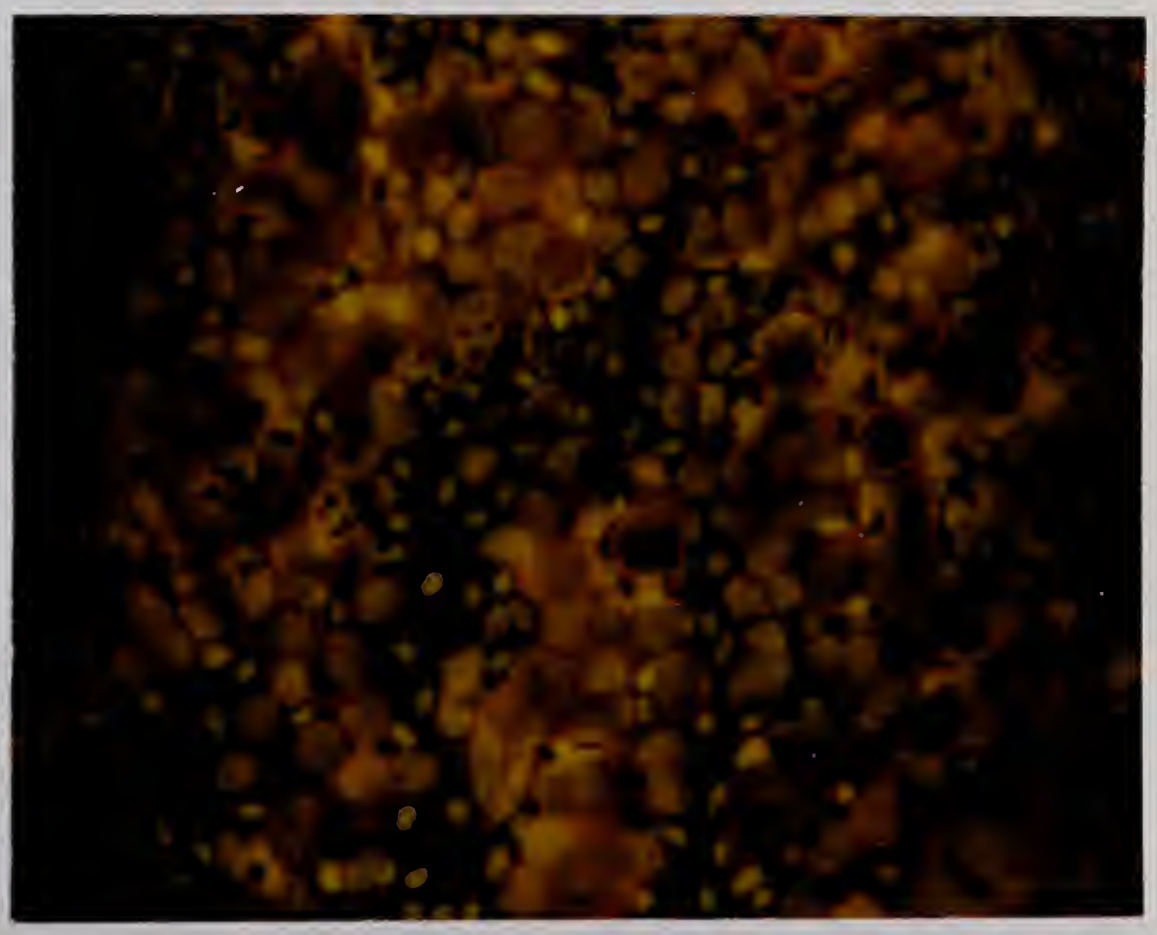
Photograph 35 was taken on High Speed Ektachrome film with an exposure time of 120 seconds, using the Reichert filters described in Materials and Methods, page 26. Photograph 37 was a 60 second exposure using the Leitz filters described on the same page. Photographs 38, 39 and 40 were 4 second exposures made with the Leitz equipment but substituting a BG-3 exciter filter for which the transmission characteristics are not yet available. The colour reproduction in the last four photographs is slightly distorted; in the microscope the nuclei appear more green and the cytoplasm more orange.

ACRIDINE ORANGE FLUORESCENCE



5 mu

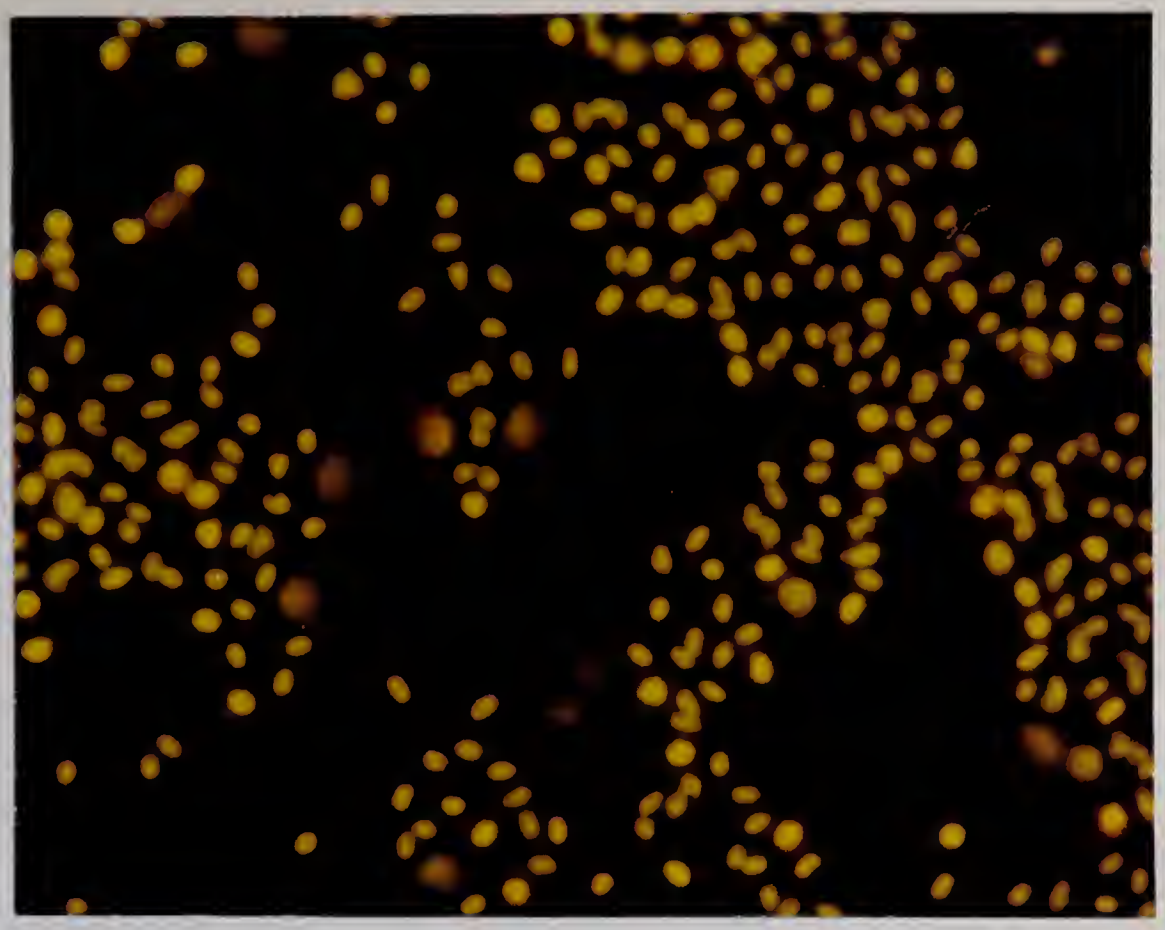
Photograph #35



5 mu

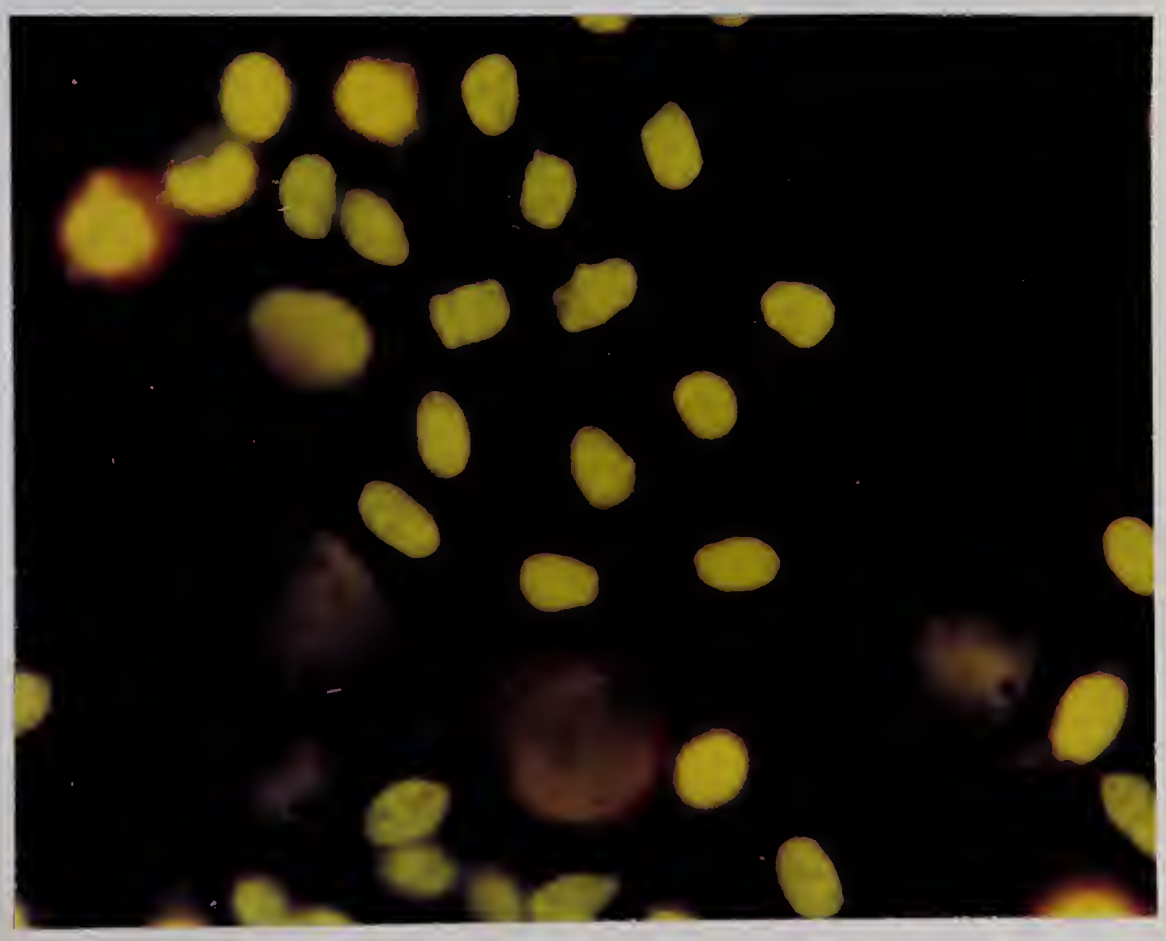
Photograph #36

ACRIDINE ORANGE FLUORESCENCE



5 mu

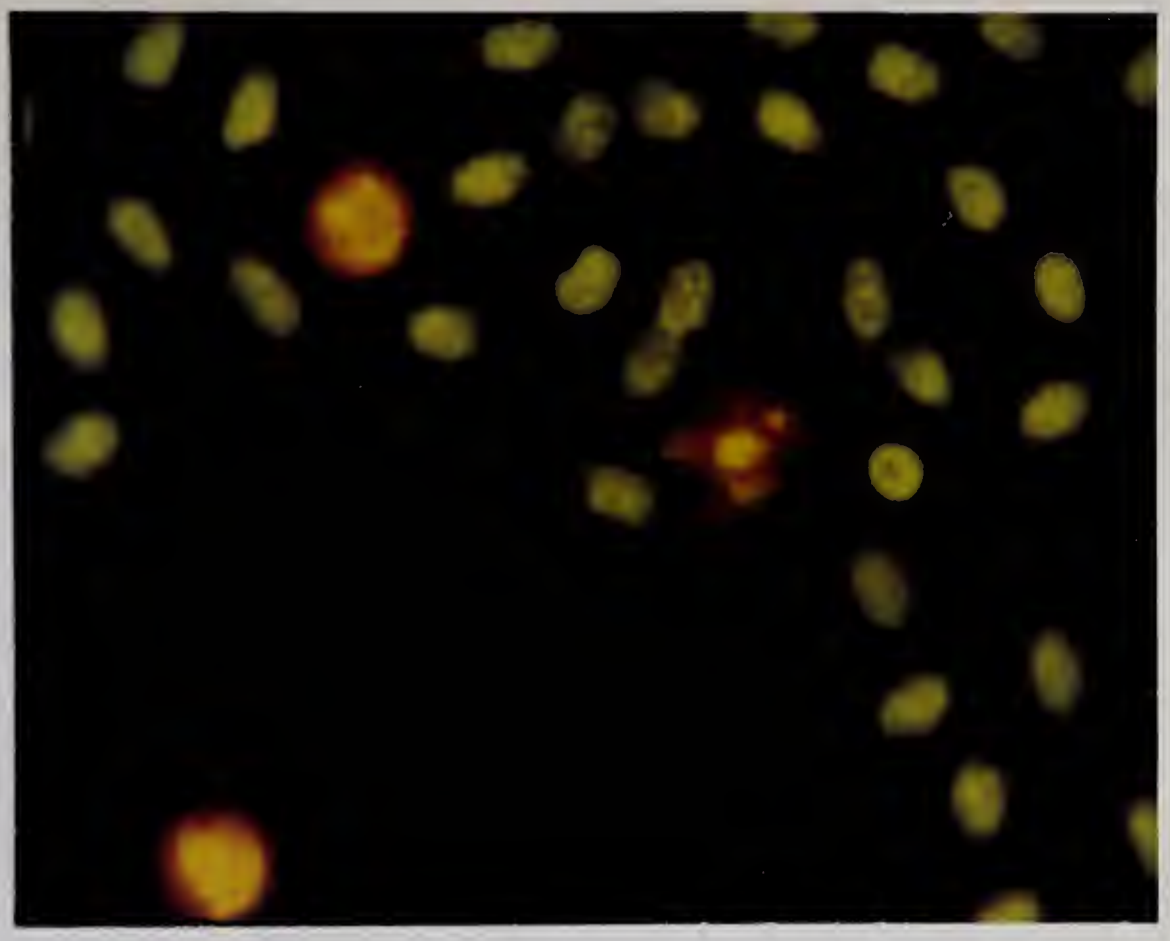
Photograph #37



5 mu

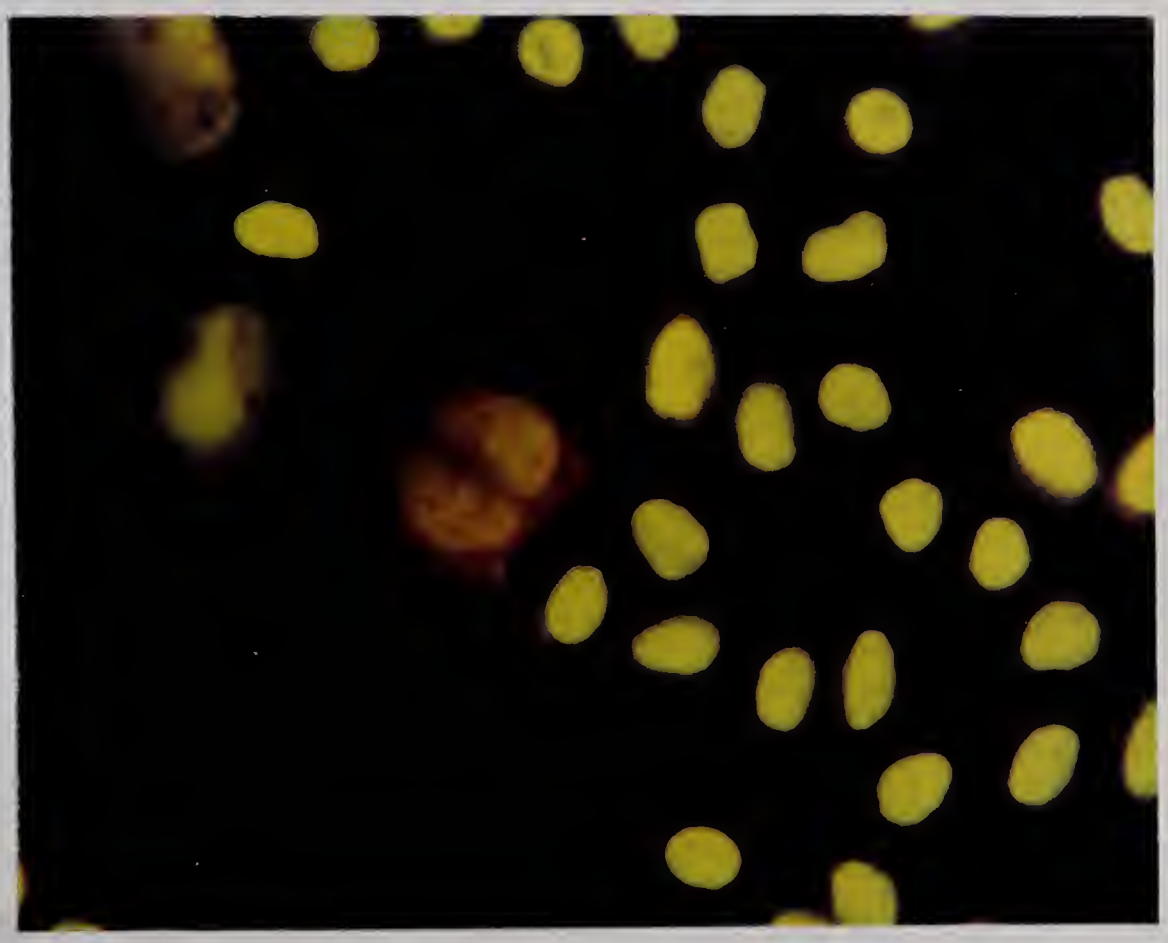
Photograph #38

ACRIDINE ORANGE FLUORESCENCE



5 mu

Photograph #39



5 mu

Photograph #40

2. Non-specific Reactions

A specific immunofluorescent reaction is one which occurs when a fluorochrome labelled antibody becomes attached to the homologous antigen to form an antigen-antibody complex. Non-specific reactions can occur in a variety of situations and give misleading results. Frozen sections of tissue, either unfixed or fixed in alcohols, are known to have an electrostatic affinity for serum proteins (Mayersbach, 1959) which varies in intensity with different tissue components. Another factor which is important in causing non-specific fluorescence is adsorption and trapping of fluorescent proteins in the exposed cytoplasmic surface, which is an inherent feature of sectioned material.

Live cells in suspension may ingest fluorescent proteins by phagocytosis. Damaged cells, either in suspension or imprinted, may become permeable and be impregnated with fluorescence. (Möller, 1960). Granulocytes and histiocytes often are a source of confusion as they exhibit bright, well organized non-specific fluorescence, the reason for which is unknown. (Nairn, 1964).

Autofluorescence of tissue or cellular components, which is not due to the presence of fluorescent dye, can also be mistaken for a specific reaction. However, this was a minor problem in this study as the autofluorescence encountered was of a different colour from the fluorescence of fluorescein or rhodamine dyes. Three types of autofluorescence were encountered; silver gray or blue fluorescence of tissue stored for a few days or longer, yellow to brown fluorescence of fresh frozen sections and the pale green fluorescence of keratinized material such as the outer layer of skin, feather follicles and shafts. This latter colour could be distinguished from fluorescein fluorescence without difficulty.

Non-specific reactions were differentiated from specific attachment of fluorescent labelled isoantibody to B isoantigens by two methods. Test materials, whether frozen sections, cell suspensions or imprints were prepared from chickens

of three B genotypes: B^2B^2, B^2B^{14} and $B^{14}B^{14}$. Fluorescent globulin fractions of isoantisera were prepared from two samples, treated in the same manner throughout. One sample was anti-B_2 serum labelled with FITC, the other anti-B_{14} serum labelled with RB200. A third sample of "non-immune serum" from a chicken not immunized against any B antigen and labelled with FITC was treated in the same manner as the isoantisera. A specific reaction was one in which the globulin fraction of the isoantiserum reacted with the test materials containing the homologous isoantigen but did not react with the control. Further, the same reaction could not be attained with either the opposite isoantiserum or non-immune globulin fraction.

Isoantiserum,labelled with fluorescent dye, either unfractionated or chromatographed on Sephadex G-200 was used throughout. The term anti-B globulin will be used to mean aliquot 2 of chromatographed isoantiserum, non-cross-reactive with B_2 or B_{14} cells unless otherwise stated.

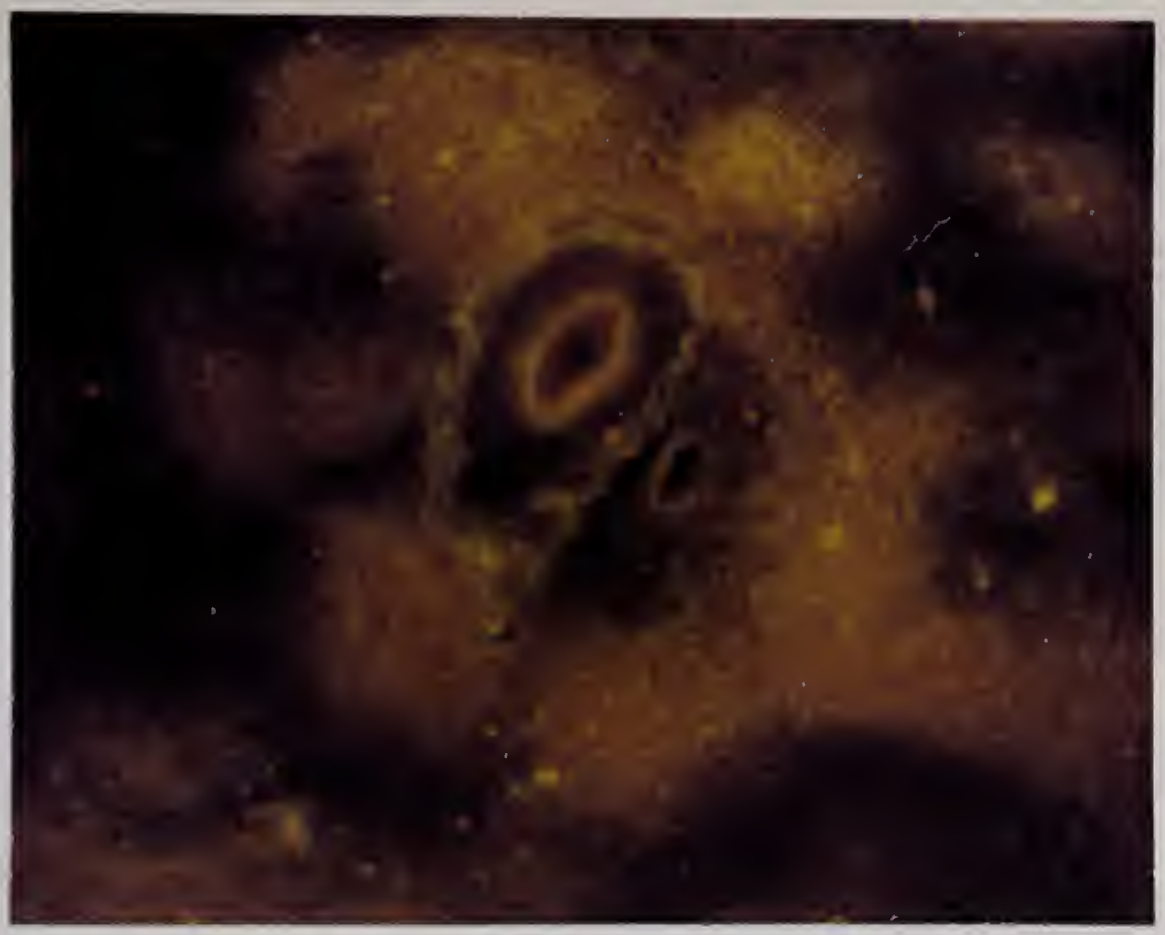
Photograph 41 is taken from a non-fixed frozen section of fourteen day embryonic spleen (B^2B^2) which has been exposed to non-immune FITC labelled globulin. The ovoid structure in the centre is a capillary in cross section, the outer wall material showing green non-specific fluorescence. The surrounding tissue shows the yellow-brown auto fluorescence of fresh, frozen sections. Photograph 42 is of the same material fixed for 10 minutes in 95% ethanol before conjugation with the fluorescent proteins. The brightly fluorescing structure is a diagonal section of a capillary in which the wall material shows brighter non-specific fluorescence. The surrounding tissue shows low-level,non-specific attachment of fluorescent protein. The bright, well organized, non-specific fluorescence of skin components may be seen in Photograph 43. The material was a frozen section of two day old chicken skin (B^2B^2) conjugated with non-immune FITC labelled globulin. Similar non-specific reactions were obtained with anti-B_2 globulin, rabbit anti-chicken globulin and horse anti-rabbit globulin, all FITC labelled. The bright non-specific fluorescence

was attributed to electrostatic adsorption of fluorescent proteins to the section. The low-level fluorescence which was present in all tissues was assumed to be due to physical trapping of the fluorescent proteins in the tissue. The bright fluorescence was noted also in cartilage and feather follicles.

Photograph 44 depicts non-specific fluorescence of leukocytes which have become permeated with fluorescent protein. $B^{14}B^{14}$ blood cells were washed in Alsever's solution and conjugated with aliquot 2 of an anti-B_2 serum chromatographed on Sephadex G-200. The accompanying phase contrast photographs of the same field demonstrates that not all cells have taken up fluorescent protein. Arrows, on Photograph 45, indicate the cells which are fluorescing in Photograph 44. An erythrocyte may be seen in the upper left corner.

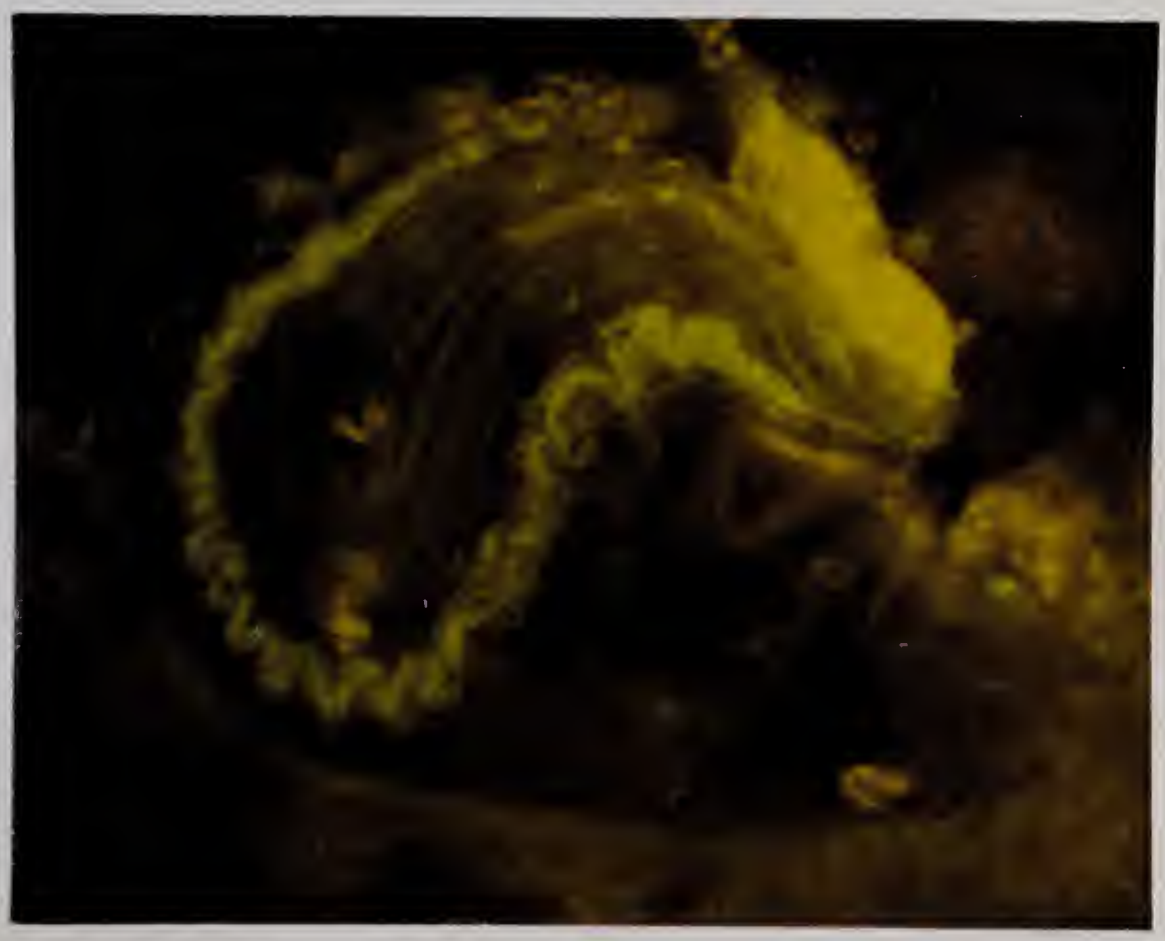
Impurities in the cell suspension, such as dust particles, often adsorb fluorescent antibodies and fluoresce brightly as in Photograph 46. Examination of the same field with phase contrast equipment can resolve the source of fluorescence as in Photograph 47, in which the arrow indicates the fluorescing dust particle. The large nucleated cells are erythrocytes, the smaller cells are leukocytes. These photographs were taken from the same material as numbers 44 and 45.

NON-SPECIFIC FLUORESCENCE



5 mu

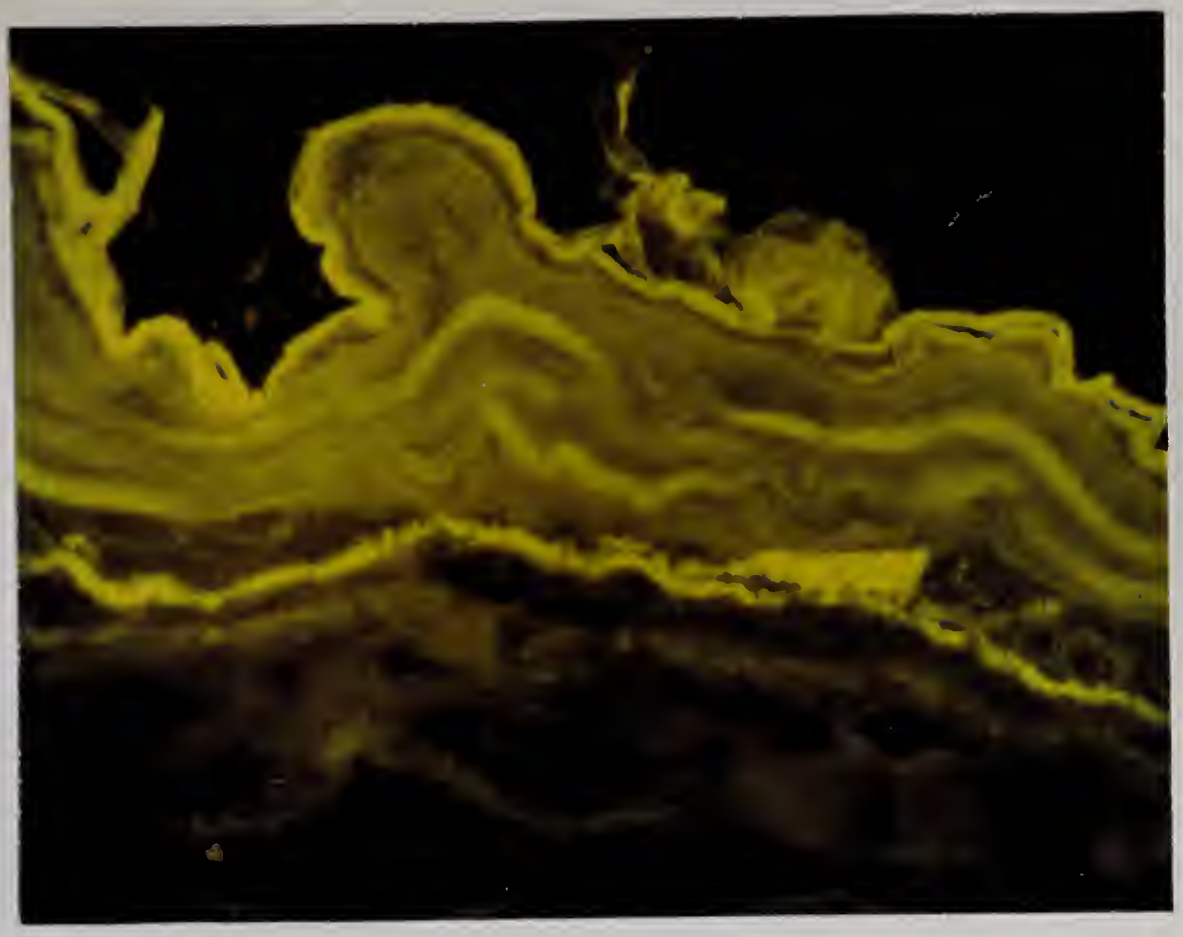
Photograph #41



5 mu

Photograph #42

NON-SPECIFIC FLUORESCENCE



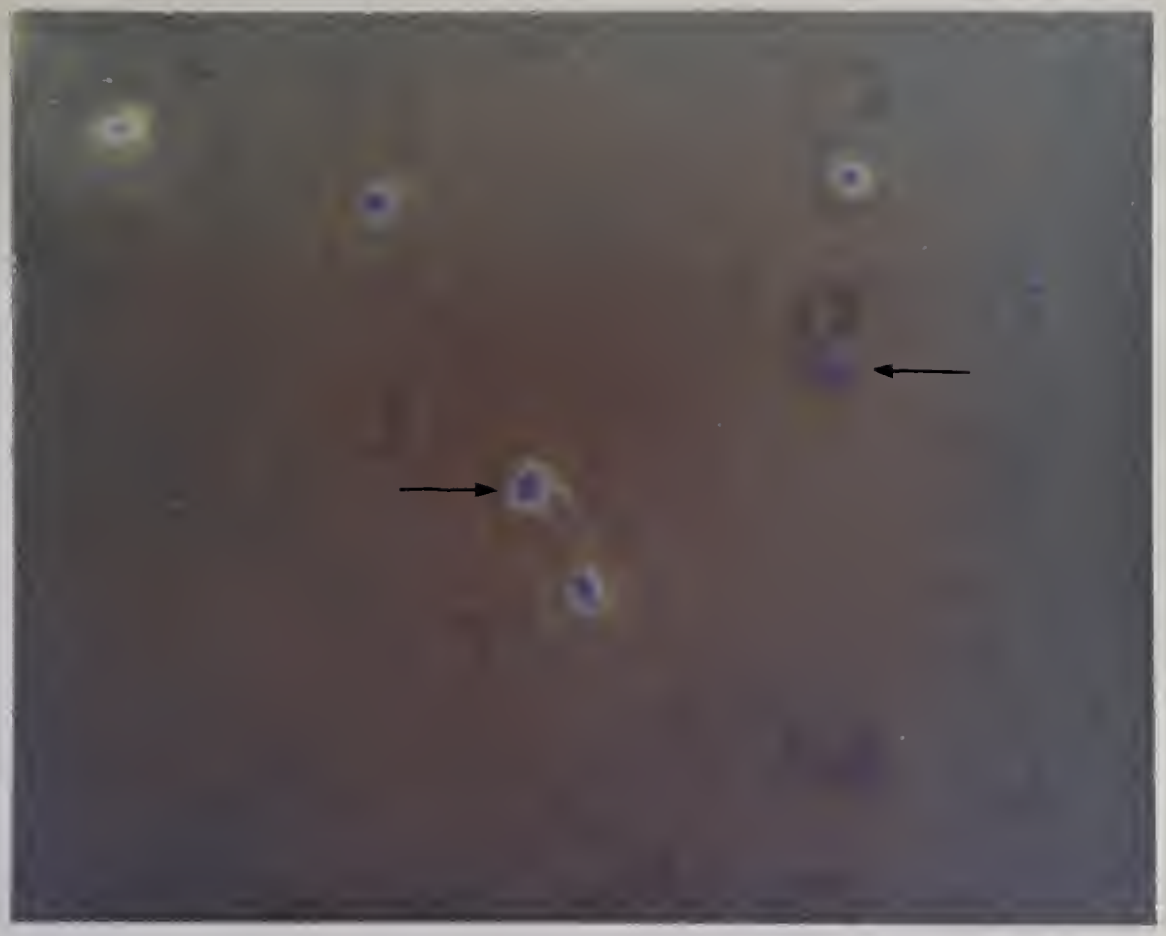
5 mu

Photograph #43

5 mu

Photograph #44

NON-SPECIFIC FLUORESCENCE



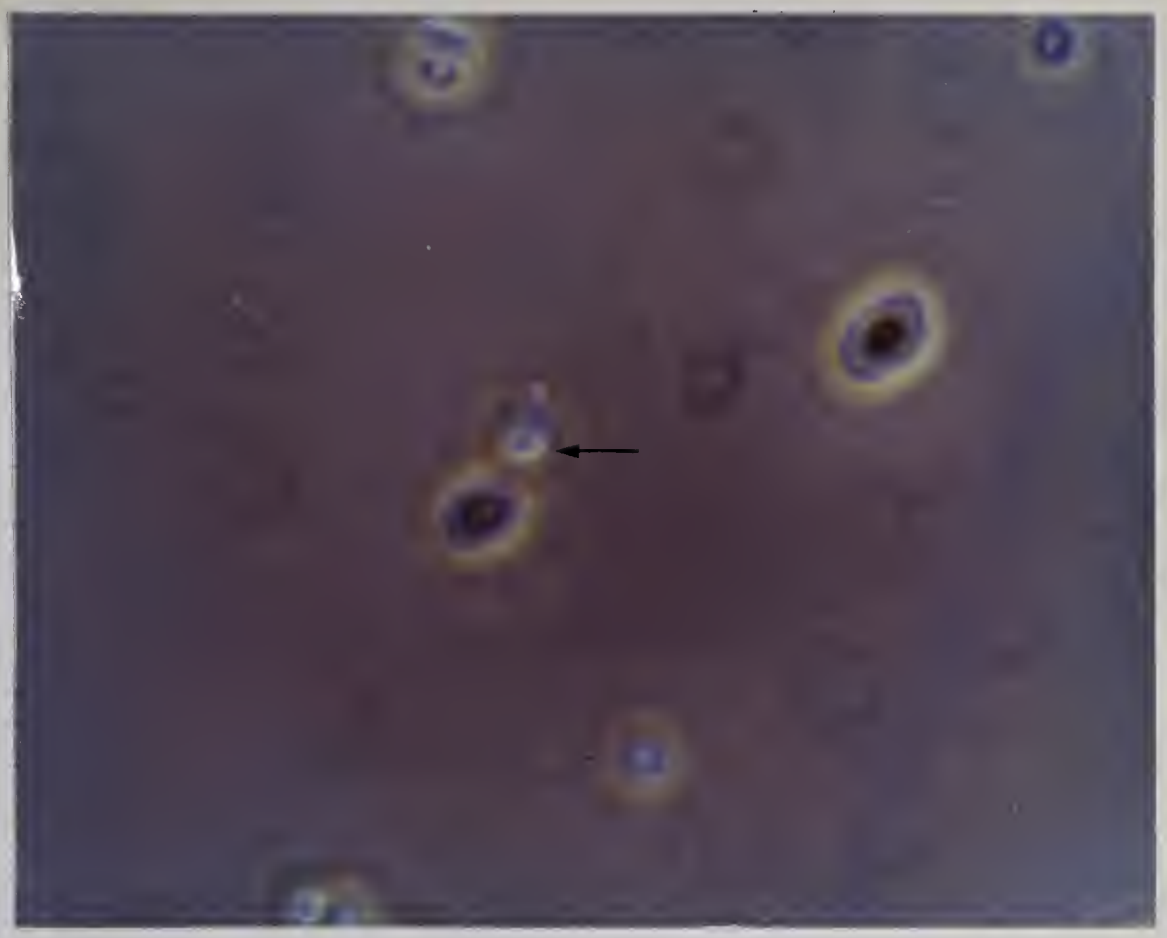
5 mu

Photograph #45

5 mu

Photograph #46

NON-SPECIFIC FLUORESCENCE



5 mu

Photograph #47

3. Specific Fluorescence

(a) Cell Suspensions

Photographs 48 and 49 illustrate the first specific reactions obtained, together with some non-specific reactions. Visualization of isoantibody attachment was enhanced by the "triple sandwich" method in which three layers of antibody are attached to the cells. The cells are $B^{14}B^{14}$ blood cells washed three times in 0.88 % saline solution. A 0.1 ml. sample of packed cells were suspended in 0.9 ml. of saline solution in a 10 ml. test tube. A 0.1 ml. sample of unfractionated, unlabelled anti-B_{14} serum was added and the cell-serum mixture agitated for one hour. The cells were centrifuged and rewashed. Agglutinated clumps were broken up by shaking on a Vortex mixer and the cells again suspended in 0.9 ml. of saline. A 0.1 ml. sample of rabbit anti-chicken globulin was added and the mixture agitated for one hour. The washing and suspension procedure was repeated. A 0.1 ml. sample of horse anti-rabbit globulin (fluorescein labelled) was added and agitated for one hour. Finally, the cells were rewashed and wet mounts prepared by placing a drop of cell suspension under a coverslip. Controls consisted of B^2B^2 cells treated in the same manner and $B^{14}B^{14}$ cells to which the initial serum applied was non-immune chicken serum. The control cells did not agglutinate or contain cells coated with fluorescent antibody, which outlines the cell surface. This is the "ring reaction" described by Möller (1960) and is a specific reaction. Arrow 1 indicates another type of specific reaction encountered with agglutinated cells, which are often associated with accummulations of fluorescent protein. Following photographs demonstrate this more clearly. Arrows numbered 2 and 3 point to leukocytes which have non-specifically adsorbed fluorescent protein. Arrow 4 indicates a non-specifically fluorescing granulocyte as described by Nairn (1964). Reactions of the type numbered 2, 3 and 4 were also observed in the control suspensions.

These photographs differ from the other colour photographs presented in that they were taken with Kodacolor X film (ASA 60) with a 4 minute exposure time. The Leitz microscope, employing a UG5/1 mm. exciter filter, and transmitted ultra-violet light only, was used.

By using simultaneous incident and transmitted illumination, we could photograph the fluorescence of cells agglutinated with a globulin fraction of iso-antiserum followed by enhancement of fluorescence with fluorescein labelled rabbit anti-chicken globulin. This is often referred to as the "double sandwich" method. Photograph 50 demonstrates the large amounts of fluorescent protein sometimes associated with agglutinated cells.

B^2B^2 cells were prepared as described in Materials and Methods in a 10% suspension and agglutinated with an equal amount of fluorescein labelled anti B_2 globulin. Examination showed weak fluorescence of agglutinated clumps. Addition of rabbit anti-chicken globulin enhanced the fluorescence to where it could be photographed. Kodak Tri-X film (black and white, ASA 200) was employed for both the fluorescence and phase photographs of the same material. The exposure times were 2 minutes for Photograph 50 with an exposure meter reading of 7 photovolts and 1/2 second for Photograph 51 with an exposure meter reading of 500 photovolts. Agglutinated cell clumps could be divided into three categories: erythrocytes only (Photograph 51), leukocytes only (Photograph 53) or clumps of mixed erythrocytes and leukocytes. (Photograph 56).

Photograph 52 shows the appearance of B^2B^2 leukocytes agglutinated with fluorescein labelled anti-B_2 globulin without the use of enhancing anti-chicken globulins. "Ring reactions" could sometimes be seen in these preparations but were too weak to photograph. Photograph 53 is of the same cells under phase contrast and Photograph 54 is a double exposure of fluorescence and phase contrast. The fluorescent and phase contrast images are slightly out of alignment because a 32X

objective was used for fluorescent photography and a 45X objective for phase contrast. The two large oval cells in the centre are erythrocytes.

Photographs 55 and 56 are also of B^2B^2 blood cells agglutinated with fluorescein labelled anti-B_2 globulin. The arrows indicate erythrocytes. Other cells in the clump are leukocytes.

Photographs 53, 54, 55, and 56 were taken on High Speed Ektachrome film (ASA 120) using a Lietz microscope with UG5/1 mm. exciter filters in the incident and transmitted light paths. Exposure times were 120 seconds and 2 seconds for fluorescence and phase contrast respectively. Exposure meter readings were 10 and 500 photovolts.

B^2B^2 Erythrocytes that had been dislodged from clumps of cells agglutinated with anti-B_2 globulin, labelled with FITC, could often be seen to exhibit a faint "ring reaction" after prolonged conjugation. If rabbit anti-chicken globulin (FITC labelled) was added and conjugated for 1 to 4 hours, the fluorescence was enhanced markedly. As Photographs 57 and 58 demonstrate, cells are outlined sharply with fluorescence which was often concentrated into brighter areas on some portions of the cell surface. All cells in these photographs are erythrocytes.

(b) Frozen Sections and Imprints

During the course of this work, approximately five hundred frozen sections were conjugated with fluorescent isoantisera and examined. These included both embryonic and adult material. Organs examined were: bursa, thymus, liver, heart, spleen, caecum, ovary and testis. Tissues included skin and muscle. Both unfixed sections and sections fixed in 95% ethanol were used. Many non-specific reactions were observed, as described earlier, but no reactions that could definitely be proven specific were found using this type of material.

As an alternative method of preparing test materials, organ imprints were made. These included bursa, thymus and spleen. Definite "ring reactions" were observed in unfixed spleen imprints. This fluorescence could be enhanced using the "sandwich technique" and photographed. Ethanol fixed spleen imprints did not show this fluorescence. However, if the spleen imprints were fixed in formalin vapour the fluorescence was brighter and more condensed. It could be photographed without the use of enhancement by the "sandwich method". Formalin fixed frozen sections of spleen also contained cells which fluoresced specifically when labelled with anti-B_2 globulin, and could be photographed. Formalin fixation also sharply reduced non-specific low level background fluorescence. Such specifically fluorescing cells were not found in bursa and thymus imprints.

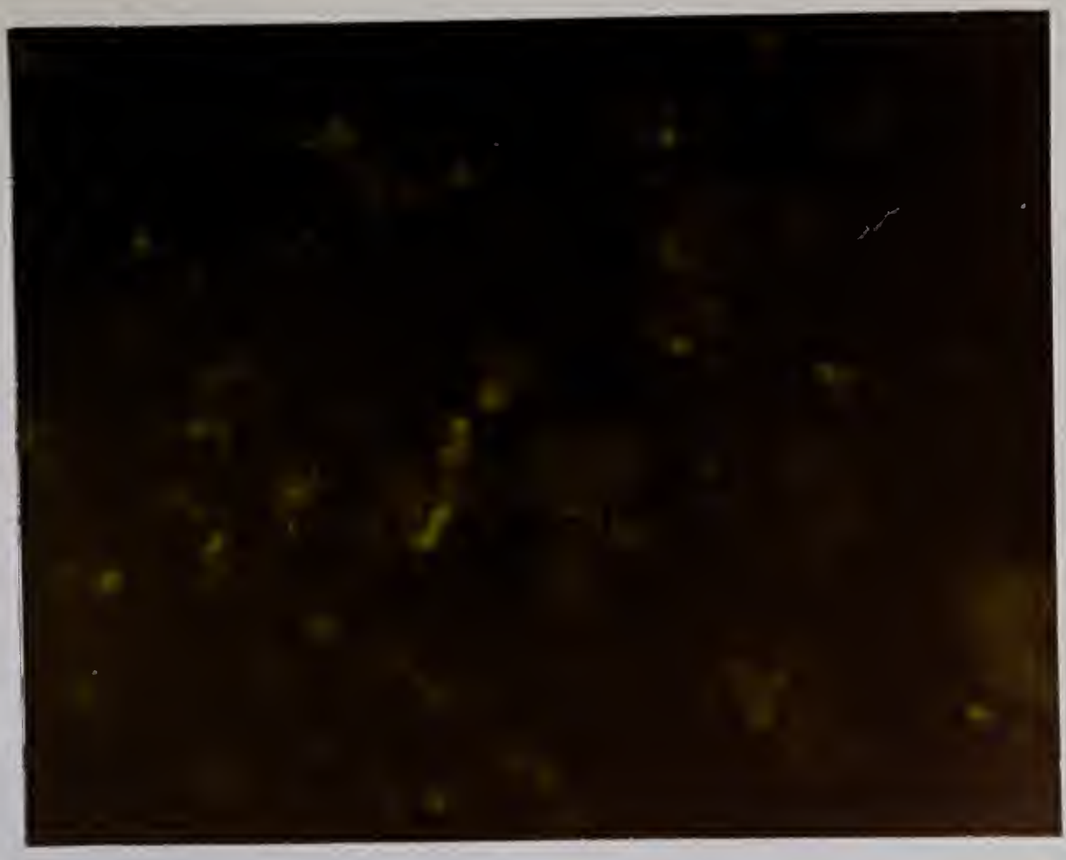
Photograph 59 shows specifically fluorescing cells in an unfixed spleen imprint. Adult B^2B^2 spleen cells were conjugated with FITC labelled anti-B_2 globulin for 30 minutes. Cells comparable to those shown in the photograph could be seen to exhibit a ring reaction. Goat anti-chicken serum was labelled with FITC and chromatographed on Sephadex G-25 to remove free dye. This was used to enhance the existing fluorescence by a further 30 minute conjugation. The usual controls were run and were negative. The imprint was examined under phase contrast and the fluorescing cells identified as spleen cells and not erythrocytes which take much longer to label with isoantibody. The halo of fluorescence around the fluorescent cells was a common feature of this type of material. It may be due to light defraction or diffusion of isoantigen into the surrounding medium.

Similar imprints were fixed in formalin vapour for 30 minutes and treated with FITC labelled anti-B_2 globulin only. The result is shown in Photograph 60. Photograph 61 shows a frozen section of adult B^2B^2 spleen, unfixed, and treated in the same manner as the material in Photograph 59. Photograph 62 is of a

formalin fixed frozen section treated as was the imprint shown in photograph 60.

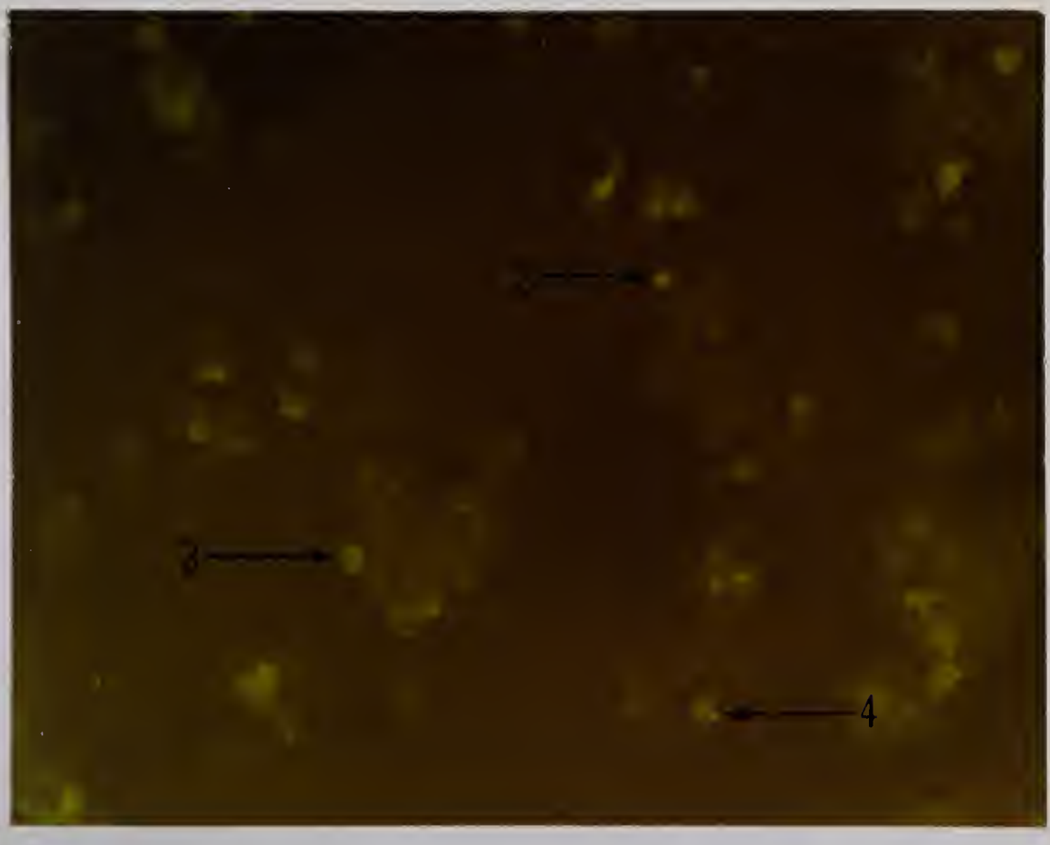
The photographs demonstrate that B_2 antigens are associated with some spleen cells. In the unfixed condition the isoantigen is associated with the cell surface. Ethanol fixation does not allow the isoantigen to be demonstrated by immunofluorescence. Formalin fixation results in enhancement and condensation of the fluorescence.

RB200 labelled anti-B_{14} globulin gave comparable results on $B^{14}B^{14}$ spleen imprints, however, Rhodamine fluorescence is much weaker than fluorescein fluorescence and photographs could not be obtained.



5 mu

Photograph #48



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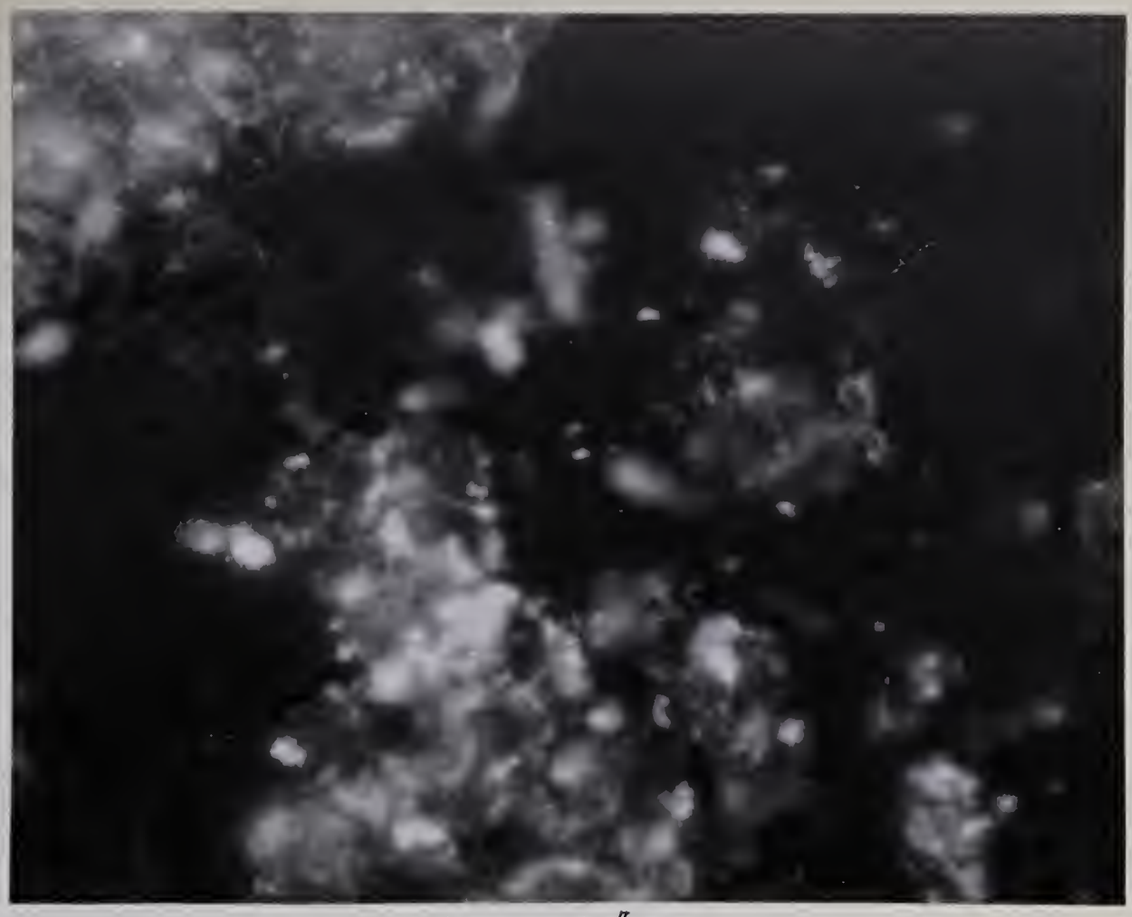
Photograph #49



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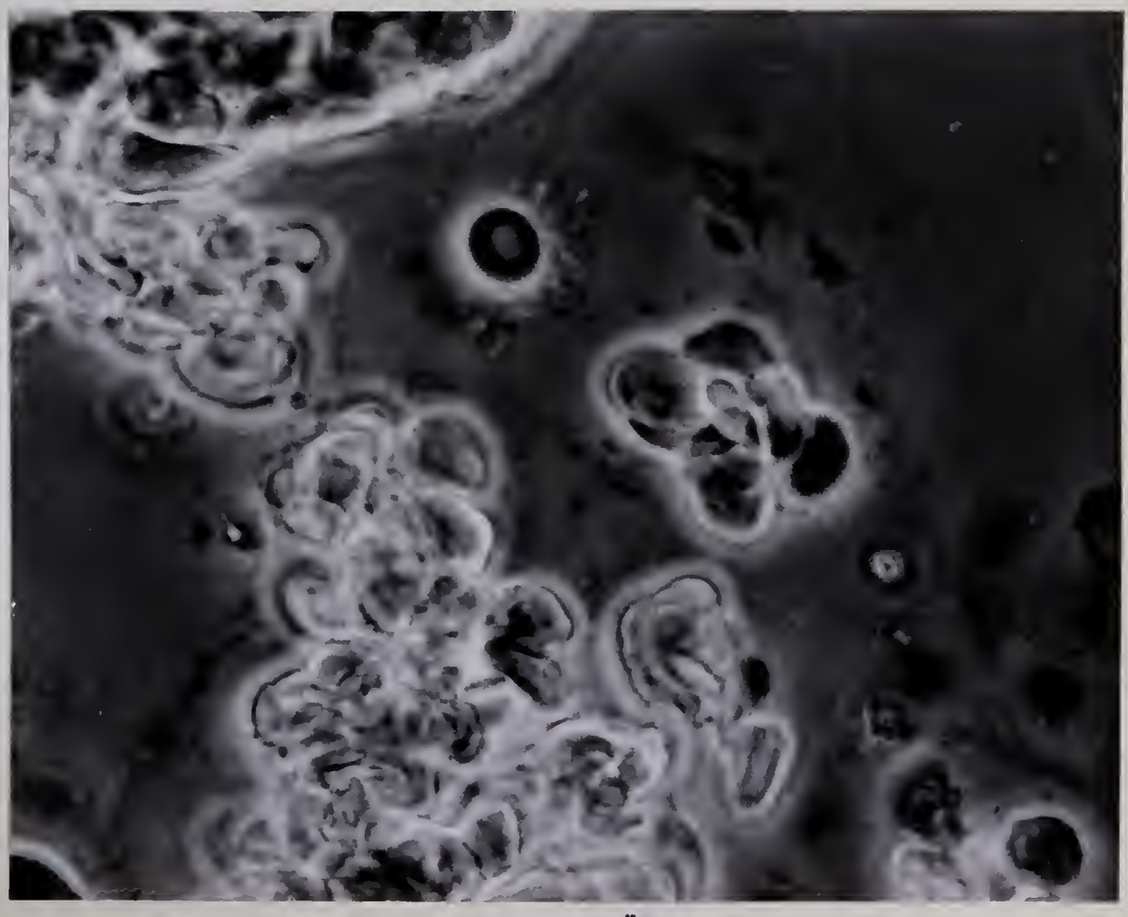
Photograph #50



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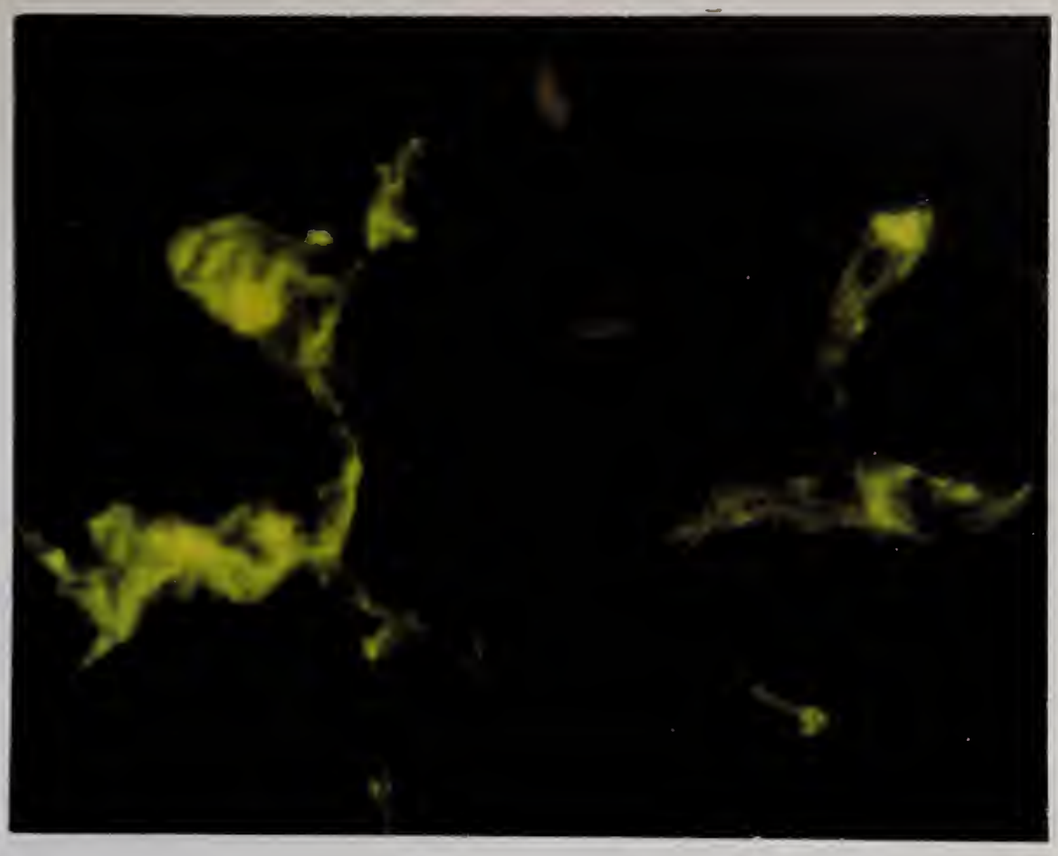
Photograph #51



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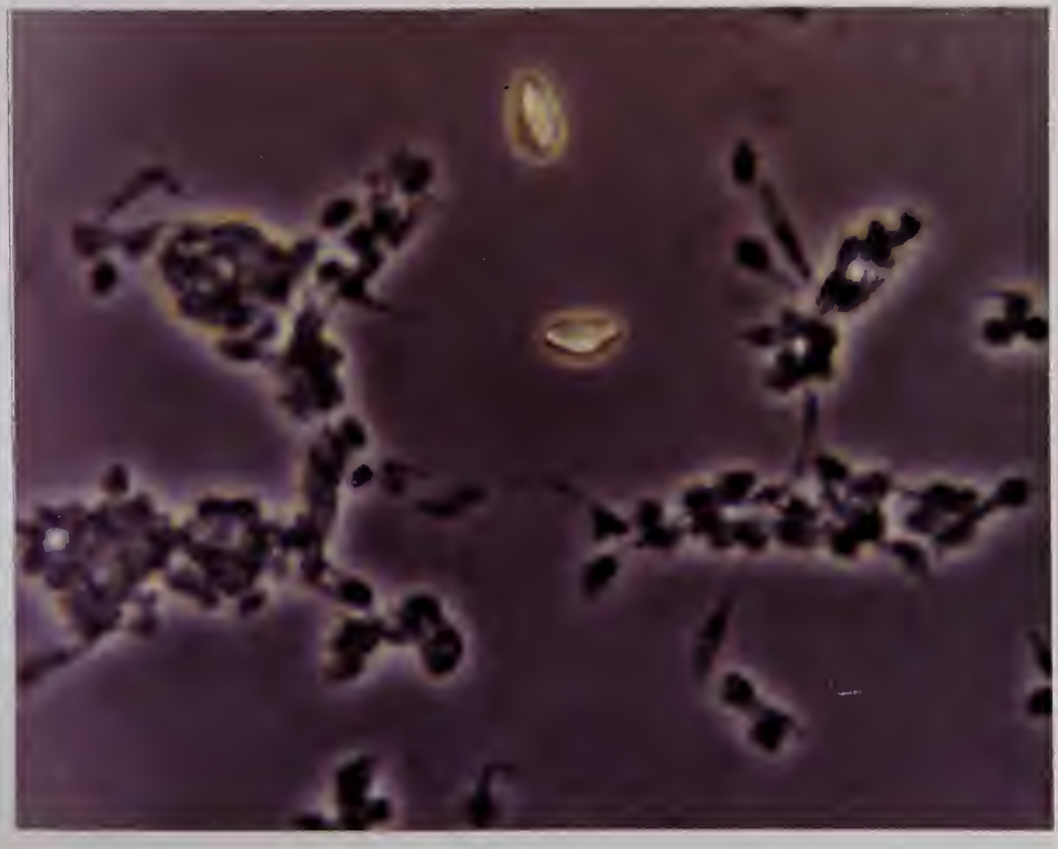
Photograph #52



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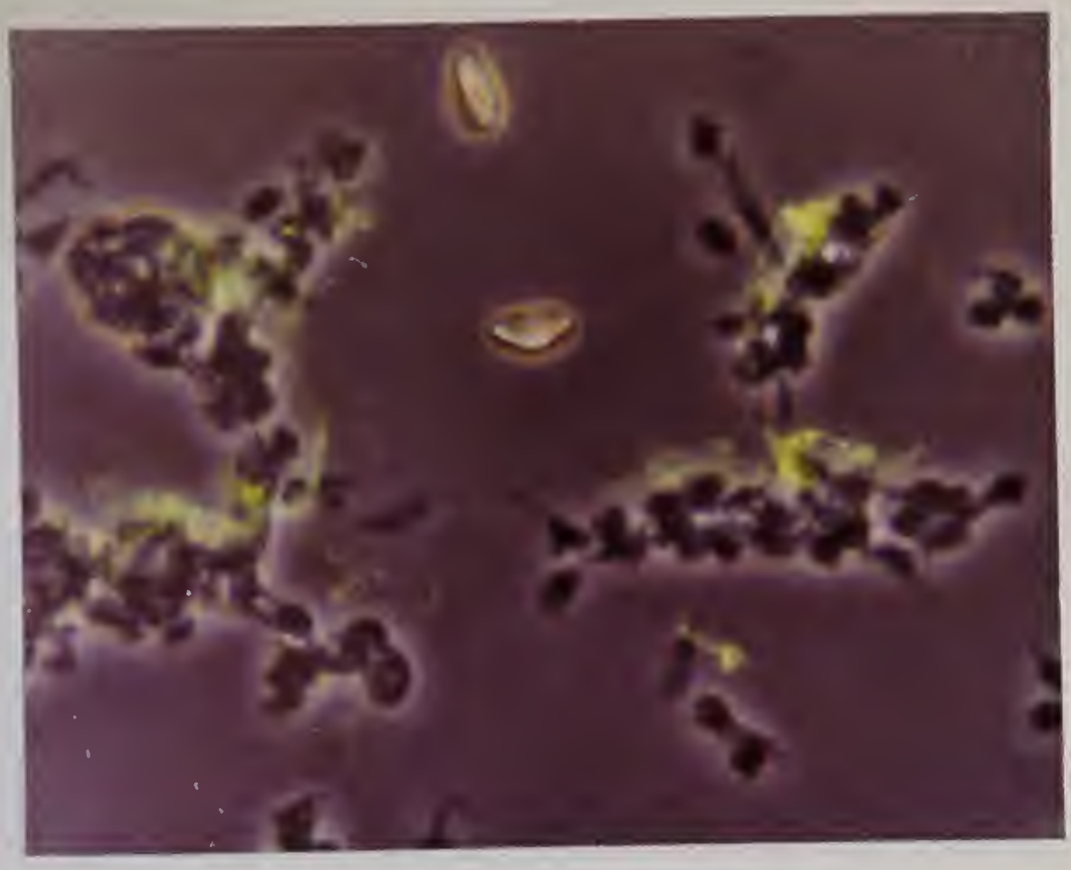
Photograph #53



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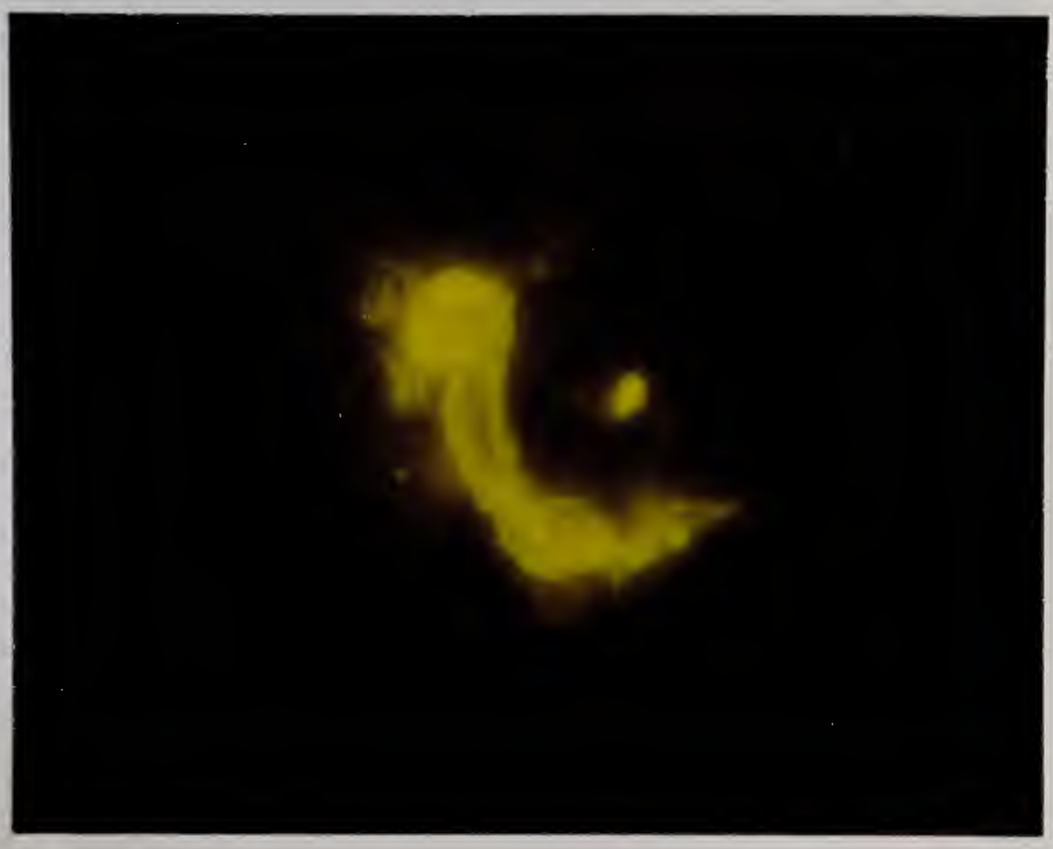

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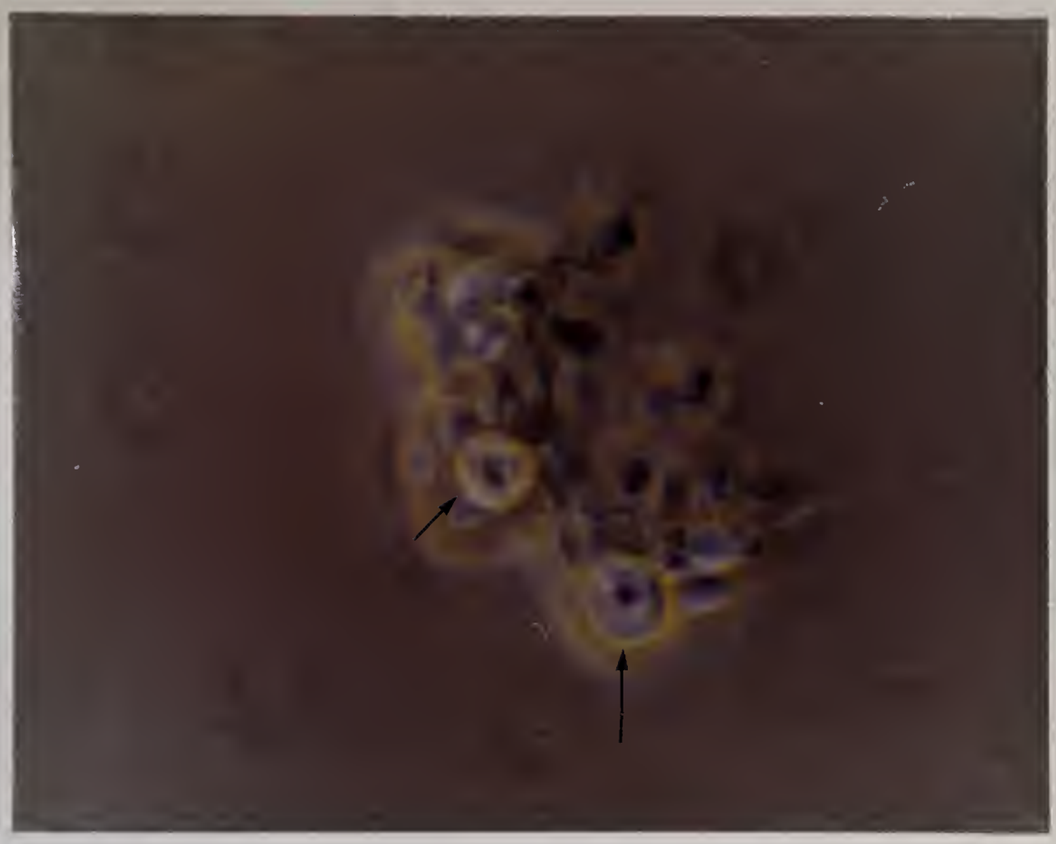
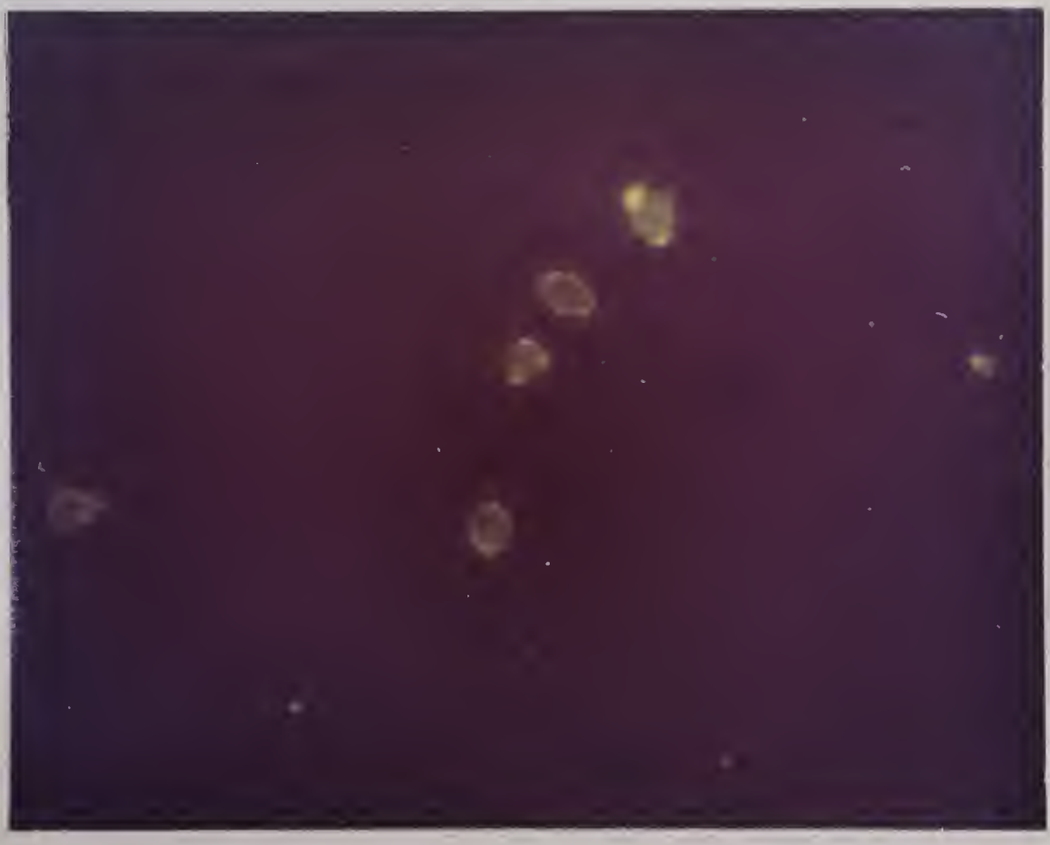


5 mu

Photograph #56



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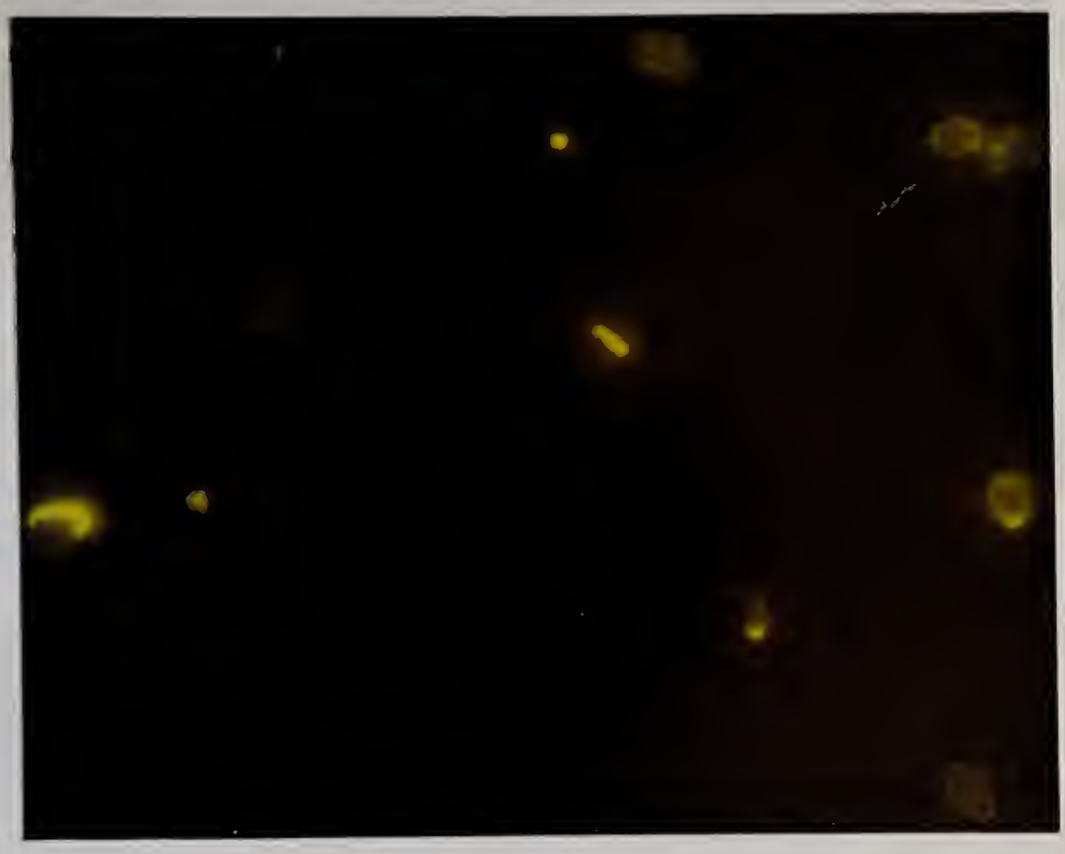

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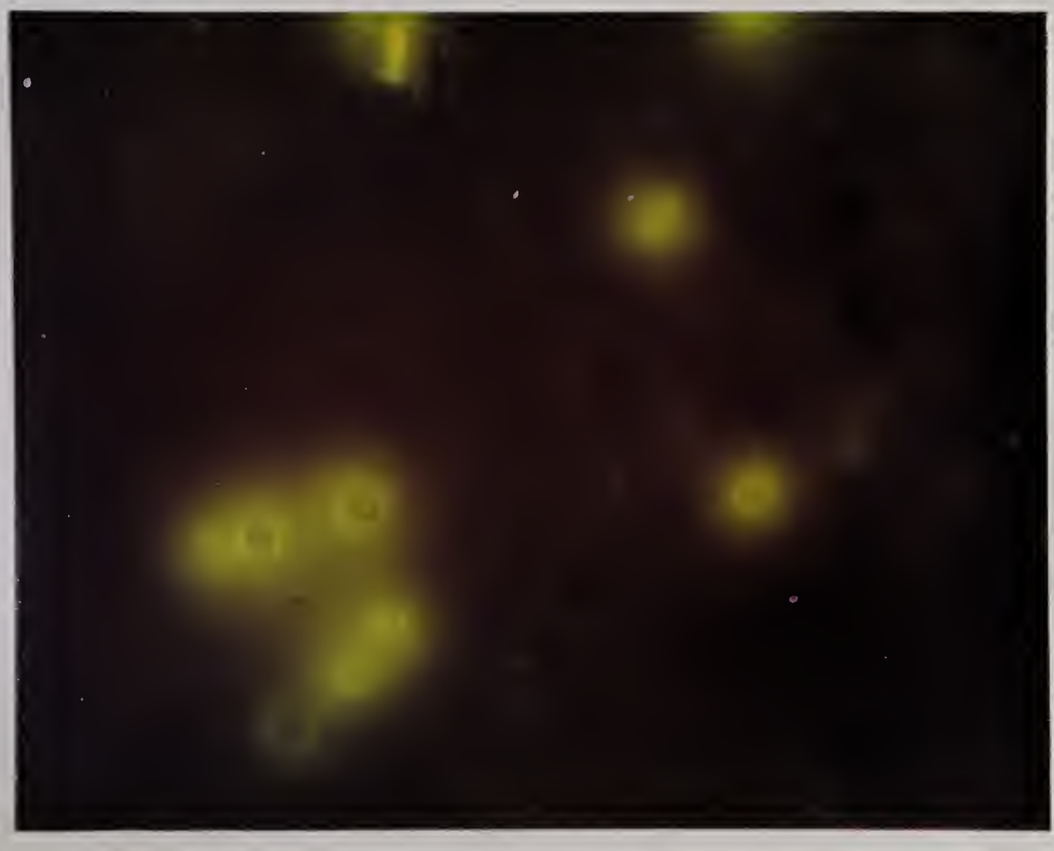
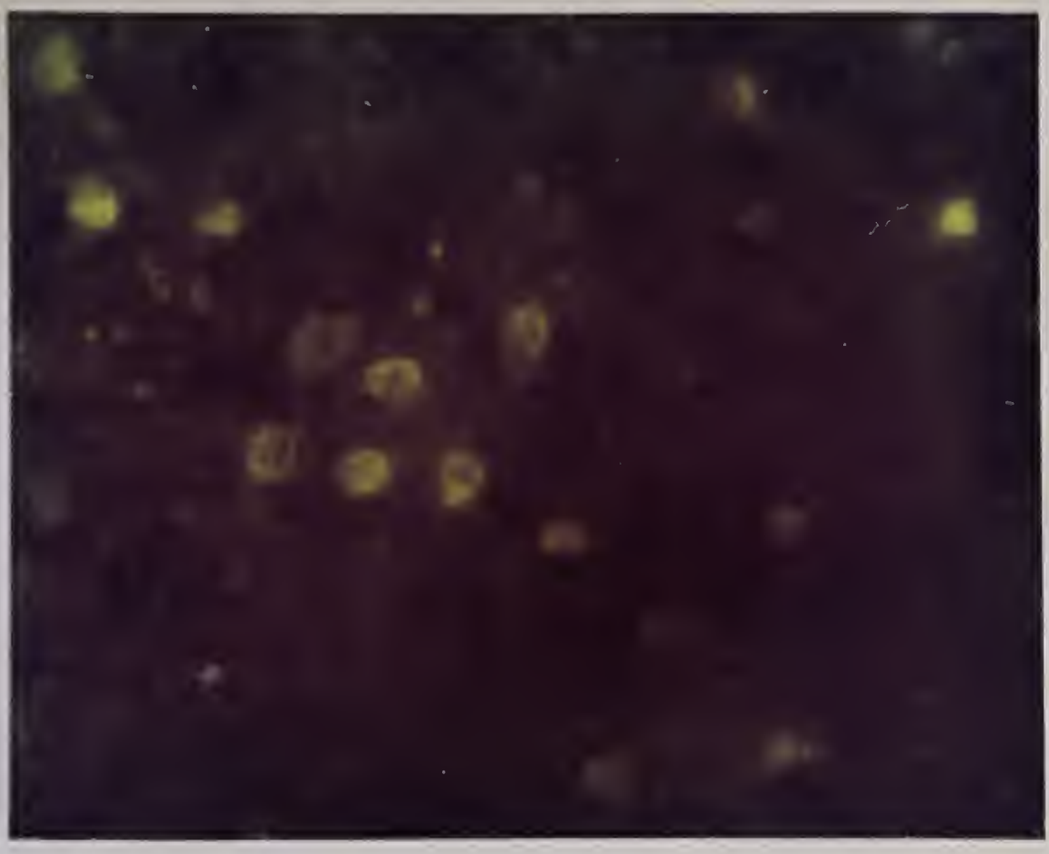
Photograph #58



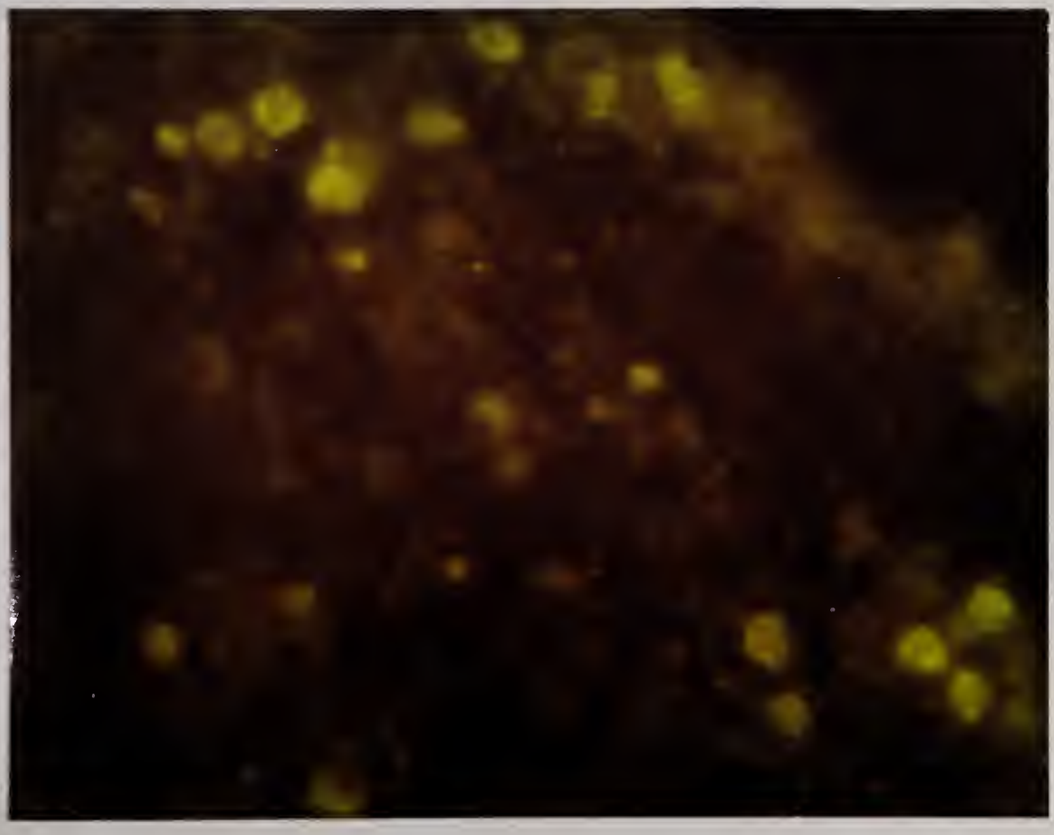
5 mu

Photograph #59



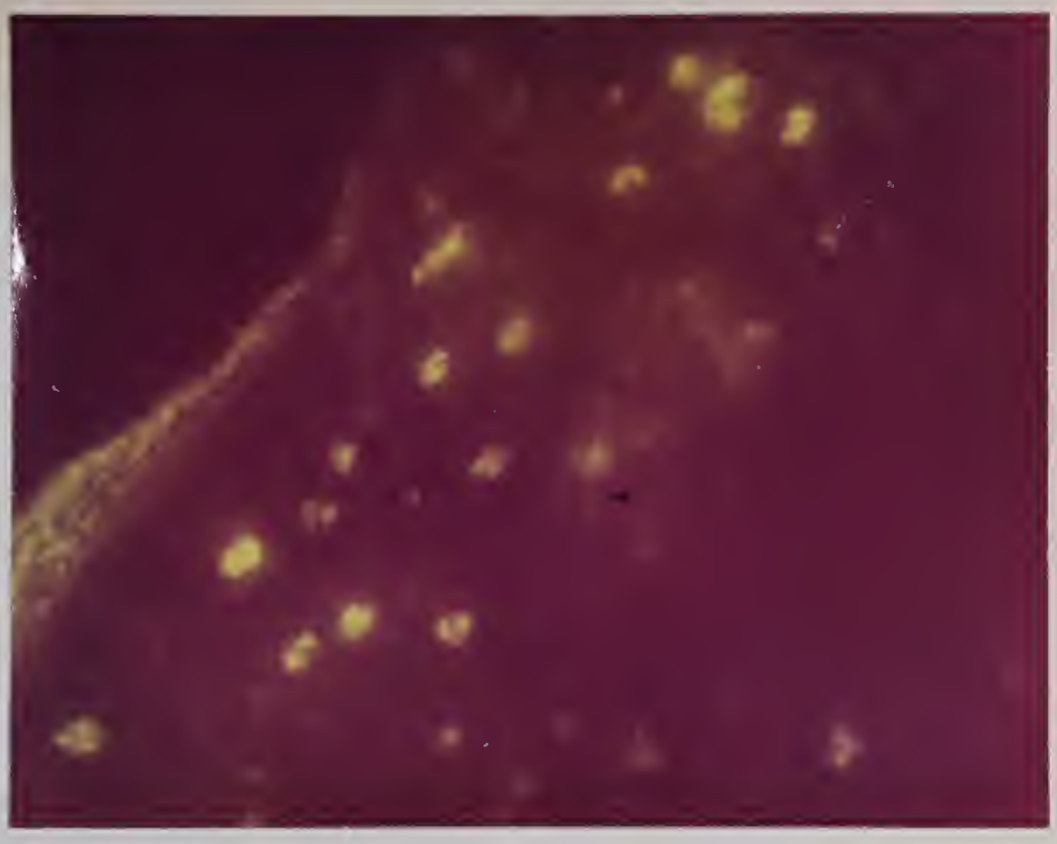
5 mu

Photograph #60



5 mu

Photograph #61



5 mu

Photograph #62

DISCUSSION

The immediate purpose of this study is to prove that transplantation isoantigens can be identified by specific immunofluorescence. The key part of this proof is the successful use of specific, fluorescent isoantibodies. The general significance of our observations is not, however, restricted to transplantation isoantigens. The isoantigens we used function as erythrocyte isoagglutinogens and as transplantation isoantigens.

Many, perhaps most, erythrocyte isoantigens do not function as transplantation isoantigens. There are, then, at least three kinds of isoantigens which are related to our observations. There are those which cause agglutination or hemolysis of red cells, those which cause the rejection of grafts between individuals of one species, and those which function in both ways. There is sufficient information about the first and the third kinds of isoantigens to justify some comparisons.

The erythrocyte isoantigens, which do not function as transplantation antigens, may mature quite rapidly. In 1933 Keeler and Castle demonstrated that rabbit red cell isoagglutinogens H_1 and H_2 are fully developed in the newborn. Human red cell isoagglutinogens A and B are fully developed long before birth. The A isoagglutinogens of chicken red cells are well developed by the seventh day of incubation and may be detected after 55 hours of incubation (Johnson L. W., 1956). On the other hand, erythrocyte isoantigens, which function as transplantation isoantigens, may mature more slowly.

In 1956, Johnson reported that the B isoantigens of chicken embryo red cells appear after seven days of incubation. These isoagglutinogens mature after hatching. In 1961 Moller extended these observations to the H-1, H-2, and H-3 isoantigens of mouse red cells. H-2 isoantigens were detected two to three days following birth and matured after six days. Moller's observations are

restricted to isoantigens which function as transplantation isoantigens, but Johnson studied two kinds of isoantigens. The A isoantigens do not function as transplantation isoantigens, but the B isoantigens are very effective transplantation isoantigens. Since the effectiveness of the B isoantigens, as transplantation isoantigens, was not reported until 1961, we reiterate some of the evidence.

In 1961 Schierman and Nordskog presented preliminary evidence that the B locus is a major transplantation locus. They exchanged skin grafts between 19 chickens of three inbred lines. Proof of the importance of the locus appeared in 1964 when Gleason and Fanguay exchanged 197 skin grafts between sibs from one inbred line. We applied adult skin grafts to 87 hybrid chicks to establish the B locus as a major transplantation locus within our flock. Additional evidence of the potency of this locus has been published (Ruth et al. 1964). In general, a skin graft bearing B isoantigens foreign to the host is rejected in half the time required for skin grafts not bearing foreign B isoantigens.

The B alleles are not, however, of equal potency. When B^1B^{13} or B^2B^{13} adult skin is grafted to B^2B^{14} chicks, rejection is acute. When B^1B^{14} adult skin is grafted the rejection is relatively mild. This suggests that B^{13} is a more potent transplantation allele than B^1. It is possible to interpret these results somewhat differently. Perhaps the B alleles of the recipient chicks play some role, but we have no evidence to justify more complex interpretations than the one we advance.

Skin grafting and specific immunofluorescence are only two of several techniques with which to study isoantigens. Another set of techniques depends upon the presence of embryo isoantigens. A form of hemolytic anemia, erythroblastosis fetalis of humans, is due to the reaction of maternal isoantibodies with paternally derived isoantigens present on fetal red blood cells. In 1953 Mitchison attempted without success to produce hemolytic anemia in newborn mice by immunization of the dam against paternal isoantigens. Experimental hemolytic anemia has been

produced in the newborn of horses, cattle, pigs, and dogs by immunization of the dam with paternal red cells, but a better experimental model of the syndrome has been described for the chicken.

In 1956 Johnson produced hemolytic anemia by immunizing hens against paternal B isoantigens. The disease begins during the last days of embryonic development when yolk proteins are known to enter the embryo's circulation. A isoantibodies are less effective than B isoantibodies despite the fact that B isoantigens mature more slowly. Perhaps those erythrocyte isoantigens which are especially effective inducers of hemolytic anemia also function as transplantation isoantigens. However this may be, the B locus of chickens provides an interesting experimental model for the Rh locus of humans. The presence of B isoantigens in chicken embryos has been confirmed by the graft versus host reaction (Ruth et al. 1965).

In general, the presence of transplantation isoantigens is detected by techniques which may be placed in two groups, immunizations and tolerance inductions. Immunization has been used to detect transplantation isoantigens in grafts and inocula used to accelerate graft rejections (Manson et al. 1964). This is an application of the difference between first and second set graft rejections emphasized by Peter Medawar. This has been extended to in vitro techniques for the detection of transplantation isoantigens in lymphocytes and other cells. The primary difference between tolerance induction and immunization is that the former usually requires the innoculation of large persistant amounts of isoantigen into recipients which are immunologically immature, repressed, or subnormal whereas the latter may be achieved with small amounts of isoantigen injected into normal recipients. The presence of transplantation isoantigens in thyroid tissue (Woodruff and Sparrow, 1956), ovary (Krohn P. L., 1958) and spleen (Billingham and Brent, 1957) has been demonstrated by tolerance induction by homogenates of these tissues. All of these techniques lend support to the generalizations that transplantation isoantigens

appear during embryonic development, mature slowly, and are present in different quantities in different tissues. Our own observations are consonant with these generalizations. Relevant to our technique, the most important observations are those of Szulman (1964) and Möller (1961).

Szulman reports that most of the endothelial and epithelial cells of six week (18 mm.) human embryos possess A, B, and H isoantigens. These isoantigens are bound to the cell walls as demonstrated by immunofluorescence. The amount of cell wall isoantigen decreased as differentiation progressed. At eight weeks (35 mm.) isoantigen was detected in secretions of the salivary glands and the stomach. Later, secreted isoantigen appeared in the gastro-intestinal tract, the respiratory system, and the pancreas. We observed immunofluorescence in the cell walls and in cytoplasmic droplets of spleen cells. These droplets resemble secret ry droplets and suggest the secretion of transplantation isoantigen. We have not studied exocrine glands.

Only one report of the demonstration of transplantation isoantigens by immunofluorescence has come to our attention. In 1961 Goran Moller applied the immunofluorescence technique to the demonstration of H-2 isoantigens on mouse cells. The purpose of this work was to explore the applicability of the method to the demonstration of H-2 and other weaker transplantation isoantigens. Moller was able to obtain specific ring reactions with a variety of living cells in suspension using the "sandwich technique".

Although the results described in this study are in essential agreement with those of Möller, this study differs in several respects. Moller prepared anti H-2 sera by injecting cell suspensions derived from tumors, spleen, lymph nodes and liver from mice of one inbred line into mice of another inbred line of a different H-2 genotype. We selected our donor-recipient combinations on an individual basis using genotyped, heterozygous birds. We injected washed blood

cells to produce B isoantisera. That Moller achieved hemagglutination titres in the order of 1000, whereas our titres were of the order of 100 is thought to be of no significance since different methods of testing for titres were used with different species.

Möller visualized the H-2 isoantigens by the "sandwich technique". That is, he applied non-fractionated unlabelled isoimmune sera to cells, followed by fluorescent rabbit antibodies specific for mouse globulins. We first visualized B isoantigens by applying fluorescent isoantibodies. The "sandwich technique" has only been used to enhance the fluorescence and to relate our observations to Möller's. Our observations are similar to Möller's, but do not depend upon the visualization of an isoantigen-isoantibody complex by attachment of a fluorescent anti globulin to that complex. In our hands the complex itself is fluorescent. Thus we avoid the chance of mistaking non-antibody globulins for isoantigen-isoantibody complexes. In other words, we are less likely to confuse the normal locations of globulins and transplantation isoantigens.

The primary technical advance of our immunofluorescence observations, beyond those previously reported, is the use of fluorescent isoantibodies. For lack of equipment we were obliged to add fluorescent label to isoantisera before fractionation. This inversion of the usual sequence permitted us to recognize and eliminate some non-specific reactions quite readily. Frozen sections have a strong affinity for chicken serum proteins, which is easily recognized when fluorescent unfractionated sera are applied to such sections. This was eliminated by chromatographic fractionation on Sephadex G-200. The diluted fractions may be concentrated by vacuum dialysis. The chicken isoantibodies survive these two steps very well, but they are irreversibly damaged by chromatographic or concentration techniques which expose them to high concentrations of salts. Concentrated, fluorescent chicken isoantibodies may react specifically with

unfixed frozen sections, but the fluorescence is somewhat diffuse and is complicated by progressive and irreversible autofluorescence. Prior fixation of frozen sections with cold ethanol caused an adsorption of fluorescent isoantibodies unrelated to their specificities. Cold ethanol has been used by others for the routine demonstration of protein antigens, but it is clearly unsuited to the specific demonstration of transplantation isoantigen. We changed fixatives.

The B isoantigens are red cell isoantigens and all red cell isoantigens which have been studied chemically are carbohydrates, not proteins. The B isoantigens are also transplantation isoantigens and transplantation isoantigens are thought to be soluble in solvents for lipids, which denature the tissue proteins without damaging either lipids or carbohydrates. We used formaldehyde as a fixative for B isoantigens on the presumption that their specific, determinative structures are not proteins, peptides or amino acids, all of which would be chemically altered by formaldehyde. It works. Precise, well-defined, specific immunofluorescence due to B_2 and B_{14} isoantigens has been observed repeatedly. Formaldehyde fixation has the additional advantage that it eliminates autofluorescence (Falck, 1965). These steps, we feel, open the way to a histochemical analysis of the transplantation antigens, and free us from the limitations imposed by the use of cell suspensions.

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